Resistance risk assessment for diamide insecticides and associated fitness costs affecting development in arthropod species with special reference to Lepidoptera

Dissertation

zur Erlangung des

Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –

der Martin-Luther-Universität

Halle-Wittenberg,

vorgelegt

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geb. 06.08.1987 in Karl-Marx-Stadt

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Verteidigt am 17.08.2017

To my family

Don't be afraid of hard work. Nothing worthwhile comes easily.

Gertrude B. Elion

Zusammenfassung

Schadinsekten aus der Ordnung der Schmetterlinge (Lepidoptera), wie die Kohlmotte (*Plutella xylostella*) und die Tomatenminiermotte (*Tuta absoluta*), zählen zu den am meisten invasiven, destruktiven Arten weltweit. Ohne angemessene Bekämpfungsmaßnahmen können Massenvermehrungen dieser Schädlinge extreme Schäden im Pflanzenbau anrichten. Daher werden Insektizide, wie z.B. Diamide, mit einem spezifischen Wirkungsspektrum gegen Lepidopteren eingesetzt. Diamide, wie Flubendiamide und Chloranthraniliprole, sind eine erst kürzlich auf den Markt zugelassene Klasse von Insektiziden, die auf den Ryanodin Rezeptor wirken. Sie erreichten innerhalb kurzer Zeit wirtschaftlichen *Blockbuster* Status und nehmen globalen Einfluss auf viele landwirtschaftliche und gartenbauliche Anbausysteme. Des Weiteren werden etablierte Insektizid-Klassen, wie die Chitin-Biosynthese inhibierenden Benzoylharnstoffe, in vielen Bereichen eingesetzt. Sie finden Anwendung als Pflanzenschutzmittel in der Forst- und Landwirtschaft sowie als Schädlingsbekämpfungsmittel in der Sanitätskontrolle. Jedoch begünstigen ineffektive Managementstrategien und zu intensiv eingesetzte Insektizid-Anwendungen die Selektion resistenter Individuen in verblüffend kürzerer Zeit.

Die molekularen Mechanismen, die der Diamid-Resistenz in *P. xylostella* und *T. absoluta* zugrunde liegen, wurden in Hinblick auf die *target-site* (Wirkort) Mutationen, G4946E und I4790M, im Ryanodin Rezeptor (RyR) untersucht. Des Weiteren wurde der Einfluss dieser Mutationen auf die Diamid-Bindung charakterisiert und diskutiert. Die Genetik sowie die funktionelle Auswirkung der kürzlich beschriebenen RyR G4946E Mutation im hochresistenten *P. xylostella* Stamm Sudlon wurden erforscht. Der Stamm Sudlon war homozygot in Bezug auf die G4946E Mutation und zeigte eine stabile Resistenz mit absoluter Kreuzresistenz zu allen kommerziell erwerblichen Diamiden. Klassische Kreuzungsexperimente ergaben, dass die Vererbung dieser Mutation rezessiv und ohne maternale Effekte ist. In Radioligand-Bindungsstudien an thorakalen, mikrosomalen Membran-Präparationen des *P. xylostella* Stamms Sudlon konnte gezeigt werden, dass die RyR G4946E Mutation funktionelle Auswirkungen auf die spezifische Diamid-Bindung sowie auf die konzentrationsabhängige Modulation der [³H]Ryanodin-Binding hat. Darüber hinaus ist die RyR G4946E Mutation von globaler Bedeutung. Die weite Verbreitung dieser Mutationen konnte durch Genotypisierung von *P. xylostella* Larven mittels Pyrosequenzierung in zehn verschiedenen Ländern mit Diamid-Wirkungsausfällen bestätigt werden. Eine Computer-Homologie-Modellierung basierend auf der Kryo-EM Struktur des Kaninchen RyR1 lässt vermuten, dass *Plutella* RyR G4946E in der Trans-Membran Helix S4 nahe der S4-S5 Verbindungsdomäne lokalisiert ist, die vermutlich in der Modulation des Spannungssensors involviert ist. Die kürzlich beschriebene Mutation

I4790M befand sich in der Helix S2 direkt gegenüber von G4946E mit einem Abstand von ca. 13 Å.

Hohe Diamid-Toleranzen wurden ebenfalls in verschiedenen *T. absoluta* Feldstämmen gefunden, die in Brasilien, Griechenland, Spanien und Italien gesammelt wurden. Dies unterstreicht die rapide Verbreitung des resistenten Phänotypen. Die Genotypisierung dieser Stämme zeigte, dass beide *target-site* Mutationen G4903E (äquivalent zu *Plutella* RyR G4946E) und I4746M vorhanden waren, allerdings in unterschiedlichen Frequenzen. Darüber hinaus wurden zwei neue Mutationen, G4903V und I4746T, in einigen resistenten *T. absoluta* Stämmen beschrieben. Der Stamm IT-GELA-SD4 aus Italien wurde mit Chlorantraniliprole unter Laborbedingungen selektiert und zeigte Synergismuseffekte bei Esterase-Inhibitoren auf. Dies deutet auf eine mögliche Involvierung dieser Enzym-Familie in der Diamid-Resistenz hin. In Bezug auf die Genetik erfolgte die Vererbung autosomal und unvollständig rezessiv, was durch eine reziproke Kreuzung des Stammes IT-GELA-SD4 mit einem sensiblen Referenz-Stamm gezeigt werden konnte. Radioliganden-Bindungsstudien an thorakalen, sarko-/endoplasmatischen Membran-Präparationen des Stammes IT-GELA-SD4 mit Individuen, die homozygot entweder G4903E, G4903V und/oder I4746M tragen, zeigten auf funktioneller Eben, dass diese Mutationen die Affinität des RyR gegenüber Diamiden ändert.

Es ist bekannt, dass Benzoylharnstoffe in der Chitin-Biosynthese eingreifen, jedoch blieb der genaue Wirkmechanismus (*mode of action*) für mehr als 40 Jahre ungelöst. In dieser Arbeit konnte somit ein altes Enigma aufgeklärt werden, indem gezeigt werden konnte, dass die Benzoylharnstoffe und andere Inhibitoren der Chitin-Synthese direkt mit der Chitin Synthase 1 interferieren. Das Gen der Chitin Synthase 1 (*CHS1*) wurde sequenziert und eine *target-site* Mutation, I1042M, wurde in dem hoch Benzoylharnstoff resistenten Stamm Sudlon-Tfm identifiziert. Die Mutation I1042M sowie die orthologe Mutation I1017F, die in der Etoxazol resistenten Spinnmilbe (*Tetranychus urticae*) gefunden wurde, wurden über eine Genommodifikation mittels CRISPR/Cas9 gekoppelt mit HDR in *Drosophila melanogaster kkv* (*CHS1* Orthologe) eingebracht. Dies führte zu hohen Resistenzgraden in *D. melanogaster* gegenüber Benzoylharnstoffen und Akariziden. Klassische genetische Kreuzungsexperimente zeigten, dass die Benzoylharnstoff-Resistenz autosomal rezessiv vererbt wurde. Darüber hinaus wurde die Mutation I1042M in anderen Populationen in Indien, China und Japan gefunden. Dies lässt vermuten, dass die Mutation unabhängig entstand.

In vielen Fällen werden Allele, die Resistenzen vermitteln, mit Fitness-Nachteilen (*fitness costs*) assoziiert und sind oftmals abhängig von Umweltfaktoren, beispielsweise der Temperatur. Aus diesem Grund wurde die Ontogenese (*life table parameters*) von drei resistenten *P. xylostella* Stämmen und einem sensiblen Stamm unter drei verschiedenen Temperaturregimen (20 °C, 25 °C und 30 °C) untersucht. Kohorten-Studien wurden im Labor durchgeführt und die beteiligten Fitness-Nachteile ermittelt. Die Temperatur von 30 °C war ungünstig für die *Plutella*

Entwicklung und hatte eine stark reduzierte Fitness in allen Stämmen zur Folge. Jedoch zeigte der sensible Stamm eine kürzere Entwicklungszeit im Vergleich zu den anderen getesteten Stämmen bei allen Temperaturen. Die resistenten Stämme zeigten signifikante Unterschiede untereinander auf und die Populationswachstumsparameter variierten sehr stark innerhalb dieser Stämme. Der Benzoylharnstoff resistente Stamm wies die höchsten Fitness-Nachteile auf, welche einen negativen Einfluss auf die Gesamt-Fitness des Stammes hatten. Dies deutet darauf hin, dass ein Selektionsdruck mittels Benzoylharnstoffen auf einen Diamid resistenten *Plutella* Stamm signifikante Effekte auf die Gesamt-Fitness und somit auch auf die Populationswachstumsparameter unter praktischen Anwendungen hat. Ergänzend wurde ein breites Kreuzresistenzprofil der resistenten Stämme erstellt und der Zusammenhang zwischen Fitness-Nachteilen und verschiedenen Resistenz-Eigenschaften von *P. xylostella* diskutiert.

Abstract

Lepidopteran pests, such as diamondback moth (DBM) (*Plutella xylostella*) and tomato leafminer (*Tuta absoluta*), are among the most destructive and invasive species worldwide. Without proper pest control strategies, mass outbreaks of these pests can cause severe damage to crop production. In order to control insect pests, insecticides are being used. Most recently, diamide insecticides acting on insect ryanodine receptors, such as flubendiamide and chlorantraniliprole, have been launched to the market and have gained blockbuster status economically with global impact in many agricultural and horticultural cropping systems. Furthermore, established classes of chemistries like benzoylphenyl ureas (BPUs) acting on chitin biosynthesis are still widely used, especially as green insecticides in agricultural crop protection, forestry and sanitary insect pest control. However, frequent insecticide application of single-components and wrong management strategies have led to high selection pressures on insects thus facilitating insecticide resistance within a short period of time.

The molecular basis of diamide resistance in DBM and *T. absoluta* was investigated with special reference to target-site mutations, G4946E and I4790M, in the ryanodine receptor (RyR) and the importance of these mutations as well as their implications on diamide binding are discussed. The genetics and functional implications of the recently described RyR G4946E mutation in the highly diamide resistant DBM strain Sudlon were studied. Strain Sudlon was homozygous for the G4946E mutation and exhibited a stable resistance with resistance ratios of >2000-fold to all commercial diamides when compared to susceptible reference strains. Classic genetic crossing experiments revealed no maternal effects and an autosomally almost recessive mode of inheritance. Radioligand binding studies using thoracic microsomal membrane preparations of DBM strain Sudlon provided direct evidence for the dramatic functional implications of the RyR G4946E mutation on both diamide specific binding and its concentration dependent modulation of [³H]ryanodine binding. Furthermore, it was shown that the G4946E RyR target-site mutation is of global importance as genotyping by pyrosequencing revealed the presence of this mutation in larvae collected in regions of ten different countries where diamide insecticides largely failed to control DBM populations. Based on a cryo-EM structure of rabbit RyR1 computational homology modelling suggests that *Plutella* RyR G4946E is located in trans-membrane helix S4 close to S4–S5 linker domain supposed to be involved in the modulation of the voltage sensor. The recently described mutation I4790M was located in helix S2 approx. 13 Å opposite of G4946E.

High levels of diamide tolerance were also found in different strains of *T. absoluta* field collected from Brazil, Greece, Spain and Italy indicating the vast spread of high diamide resistance. Genotyping for target-site mutations in the RyR of *T. absoluta* strains revealed the presence of both G4903E – equivalent to G4946E in DBM - and I4746M mutations at different

frequencies. Furthermore, two novel mutations, G4903V and I4746T, were detected in some of the resistant *T. absoluta* strains. Strain IT-GELA-SD4 from Italy was selected with chlorantraniliprole under laboratory conditions and had shown synergism effects by esterase inhibitors suggesting a possible role for this enzyme family in resistance. The genetics of resistance by reciprocally crossing the IT-GELA-SD4 strain with a susceptible strain had shown an autosomal incompletely recessive mode of inheritance. Radioligand binding studies using thoracic sarco-/endoplasmic membrane preparations of the IT-GELA-SD4 strain, largely consisting of individuals homozygous for either G4903E, G4903V and/or I4746M, provided functional evidence that these mutations alter the affinity of the RyR to diamides.

It is known that BPUs interfere with chitin biosynthesis but the exact mode of action has remained elusive for over 40 years. In this study, it was shown that BPUs and other chitin synthesis inhibitors directly interfere with insect chitin synthase 1 and the old enigma was unraveled. Gene sequencing of chitin synthase 1 (*CHS1*) was performed and a target-site mutation, I1042M, was identified in strain Sudlon-Tfm, highly resistant against BPUs. Introducing this mutation, as well as the orthologous mutation I1017F present in etoxazole resistant two-spotted spider mite CHS1, in *Drosophila melanogaster kkv* (*CHS1* orthologue) by a CRISPR/Cas9 coupled with HDR genome modification approach, provided high levels of resistance in *D. melanogaster* against both BPUs and growth regulator acaricides. Classic genetic crossing experiments were conducted in DBM strain Sudlon-Tfm and it was shown that BPU resistance was inherited in an autosomal recessive way. Furthermore, the mutation I1042M was identified in other DBM populations from India, China, and Japan suggesting an independent origin of this mutation.

In many cases resistance alleles have been associated with fitness costs and are often dependent on environmental factors such as temperature. Hence, the life table parameters of three insecticide resistant and one susceptible DBM strain were investigated under three different temperature regimes (20 °C, 25 °C and 30 °C). Cohort studies were conducted in the laboratory and involved fitness costs were estimated. In general, 30 °C was unfavourable for DBM development resulting in a reduced fitness in all strains. However, the susceptible strain had shown a shorter developmental period compared to the resistant strains at all three temperatures. Moreover, the resistant strains differed significantly between one another and the population growth parameters varied among the strains. The BPU resistant strain had shown the highest costs affecting the overall fitness of this strain which suggests that BPU selection pressure on a diamide-resistant DBM strain leads to significant effects on the overall fitness and population growth parameters under applied conditions. Additionally, the broader cross-resistance profile of the resistant strains was tested and the relation of fitness costs and different resistance traits present in DBM is discussed.

Acknowledgements

Working at Bayer has been an exciting opportunity for me to be part of the wonderful scientific community that is Bayer AG, Division Crop Science. I sincerely thank Prof Dr. Gerald Moritz and Dr. Ralf Nauen for the three years of great supervision and for pushing me into the right direction whenever I had need of it.

I would like to thank Prof. Dr. Gerald Moritz for his guidance, advice and support throughout my thesis, especially during the writing process. I am sincerely obliged to Dr. Ralf Nauen for taking me on in his group, tasking me with such an interesting subject, supervising me on a highly professional level and indeed for his encouragement. In addition, I thank Prof. Dr. John Vontas for the great collaboration work we did. Many thanks to Dr. Emmanouil Roditakis, Dr. Vassilis Douris, Dr. Thomas Van Leeuwen and Prof. Dr. Chris Bass for their part in our fruitful collaboration. I'd like to thank Dr. Markus Dollinger for his great support as well as for the nice and interesting discussions we had.

I'd like to express my sincere thanks to my work colleagues at Bayer AG, Division Crop Science, Corinna Schorn, Bettina Lüke, Annemarie Hertel, Marion Zaworra, Antje Rottmann, Marion v. Zeddelmann and Harald Köhler, for their help and support both within the lab and in the outside world. Thank you for the fun and wonderful moments. I have grown so much with you all and count you all as good friends.

Many thanks to my other Bayer colleagues: Ellias Bellis, Sabine Langner, Anja Rottmann, Maike Günther, Claudia Wehr, Gabriele Lachner, Roland Kunze and Anette Jacobs for support and good discussions.

I sincerely thank Dr. Stephanie Krüger at Martin-Luther-University Halle-Wittenberg for her advice and help throughout the past years.

I'd like to thank my master students Angel Popa Baez and Debora Boa Ventura for their feedback during my supervision.

I would like to address particular words of thanks to my family, especially to Andra and David for their enduring love, support and encouragement during difficult times. I also thank my friends who stood steadfast by my side whether they were near or far.

Table of Contents

List of Abbreviation

AA	Amino acid
Å	Angstom
BPU	Benzoylphenyl urea
cDNA	complementary deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CHS1	Chitin synthase 1
CL	Confidence limits
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSI	Chitin synthesis inhibitors
D	Degree of dominance
DBM	Diamondback moth, *Plutella xylostella*
DEF	S.S.S-tributyl phosphorotrithioate
DEM	Diethyl maleate
DNA	Deoxyribonucleic acid
EC	7-ethoxy coumarin
EST	Esterase
ER	Endoplasmic reticulum
F1	first filiale generation
Fig	Figure
g	Relative centrifugation force (g-force)
g	Gram
gDNA	genomic deoxyribonucleic acid
GST	Glutathione-S-transferase
gRNA	guide ribonucleic acid
HDR	Homology Directed Repair
IGR	Insect Growth Regulator
IRAC	Insecticide Resistance Action Committee
IPM	Integrated Pest Management
IRM	Insect Resistance Management
K_D	Dissociation constant
kdr	Knock down resistance
kkv	krotzkopf verkehrt
LC	Lethal concentration
mg	Milligram
mL	Milliliter

mM	Millimolar
min	Minute
μg	Microgram
μL	Microliter
μm	Micrometer
M	Molar
NA	1- and 2-naphthyl acetate
nm	nanometer
nt	Nucleotide
ORF	Open reading frame
P450(s)	Cytochrome P450-dependent monooxygenase(s)
PAD1	[3-iodo-N-(2-methanesulfonyl-1-methyl-ethyl)-N´-[2-methyl-4-(1,2,2,2-tetrafluoro-1-trifluoromethyl-ethyl)-phenyl]-phthalamide]
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
R&D	Research and Development
RM	Resistance Management
RNA	Ribonucleic acid
RR	Resistance ratio
RyR	Ryanodine Receptor
rpm	Revolutions per minute
s	Seconds
SNP(s)	Single nucleotide polymorphism(s)
SD	Standard deviation
SE	Standard error
SEM	Standard error mean
SR	Sarcoplasmic reticulum
WG	Water dispersible granules

List of Publication

Chapter 1

Section sections 1.2 (except 1.2.2) and section 1.4.

Nauen, R. and Steinbach, D., (2016): Resistance to diamide insecticides in lepidopteran pests. In Horowitz, A.R., Ishaaya I., (eds.), Advances in Insect Control and Resistance Management in 2016, Springer Verlag, DOI 10.1007/978-3-319-31800-4_12.

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Chapter 3

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Chapter 4

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Chapter 5

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Chapter 6

Sections 6.2, 6.3, 6.5.

Nauen, R., Steinbach, D., 2016. Resistance to diamide insecticides in lepidopteran pests. In Horowitz, A.R., Ishaaya I., (eds.), *Advances in Insect Control and Resistance Management* in 2016, Springer Verlag, DOI 10.1007/978-3-319-31800-4_12.

Chapter 1

Introduction

The Green revolution which took place between 1930s and the late 1960s has enabled world food production to double over the course of the past 50 years due in large part to the use of artificial fertilizers, pesticides, and high-yield crop varieties. This in turn has affected human population levels which have more than doubled and today numbers approximately 7.4 billion people (http://www.prb.org, 2016). The global population is projected to grow by 70 million per annum, increasing by 30 % to 9.2 billion by 2050 (Popp *et al.* 2013). Consequently, the demand for food production is estimated to increase by 70 % due to changes in dietary habits such as greater consumption of meat and milk products and a concomitant greater need of grains for livestock feed (FAO 2009). The provision of additional land for agriculture could be one strategy in order to meet this demand. However, agricultural expansion is limited as it would lead to a reduction of forests and the natural habitats of wildlife, wild relatives of crops and natural enemies of crop pests (Popp *et al.* 2013). At the same time, problems like climate change, reduction in quality of arable land and pollutions need to be tackled. Owing to these challenges modern agriculture aims at a sustainable production that also enables an increase in crop yield (productivity) on existing land by decreasing crop losses and minimizing adverse environmental impacts.

One approach is pest control by using crop-protecting chemicals, i.e. pesticides including insecticides, fungicides and herbicides, to minimize crop losses and therefore, increase agricultural output for most field, fruit and vegetable crops. In order to protect our plants, various pesticides are being used to destabilize, perturb, or inhibit crucial biochemical and physiological targets related to metabolism, growth, development, nerve and muscles, or behaviour in pestiferous organisms (Cohen 1993). An average of 35 % of potential crop yield is lost to pre-harvest pests on a global scale and without pesticides the yield loss could raise to 70 % due to pest activities (Oerke 2005). This would have a major impact on food security as food production would drop, the quality of crops would decline and food prices would soar (Popp *et al.* 2013).

Insecticides, a class of chemicals that specifically act against pest insects, play an important role in crop protection and in the reduction of disease transmission. Each year more than 1 million deaths result from diseases transmitted by insects (Sun *et al.* 2015). Furthermore, insects damage crops as well as stored agricultural products and in doing so, are accountable for substantial economic losses, worth billions of dollars annually (Talekar and Shelton 1993, Ortiz-Urquiza *et al.* 2015).

The global insecticide sales market was estimated to be worth 17.016 million $US in 2013 (Sparks and Nauen 2015) which displays the importance of insecticides usage for agricultural

practice. On the contrary, the increased usage of chemicals such as insecticides, especially conventional insecticides like organophosphates or carbamates present environmental and health risks, which can often not be predicted or foreseen (Sechser and Reber 1998). Furthermore, frequent insecticide application and wrong management strategies lead to high selection pressures on insects thus facilitating insecticide resistance (Roush 1993, Heckel 2012). As a result field failures are inevitable and due to pest activities crop losses are reported. Insecticide resistance in insect pests is an increasing problem and lepidopteran pests are among the most destructive and invasive species.

1.1 Lepidopteran pest species

The insect order Lepidoptera comprises more than 160.000 described species of butterflies and moths (van Nieukerken *et al.* 2011). Hence, they are the second most diverse insect order after beetles. The majority of adult moths and butterflies are beneficial insects as they pollinate many plants by feeding on nectar using their siphoning proboscis. On the contrary, caterpillars are equipped with chewing mouthparts and feed on various parts of the plants or act as leaf miners of succulent plant tissues which in turn can seriously damage the plant up to complete defoliation. Therefore, many lepidopterans are major pests to forests, stored grain and food crops, especially at a high population density. A few examples of agricultural pests with frequent outbreaks leading to severe damage or crop losses that need to be mentioned include: cotton bollworm, *Helicoverpa armigera* (Hübner 1808) (Noctuidae) codling moth, *Cydia pomonella* (L. 1758) (Tortricidae), striped rice stemborer, *Chilo suppressalis* (Walker 1863) (Crambidae), tomato leaf miner, *Tuta absoluta* (Meyrick 1917) (Gelechiidae), diamondback moth, *Plutella xylostella* (L. 1758) (Plutellidae).

1.1.1 Diamondback moth, *Plutella xylostella*

Diamondback moth (DBM), *P. xylostella*, is a cosmopolitan pest of brassica crops and the most widely distributed moth of all Lepidoptera (Shelton 2004). There are numerous speculations about the origin of this species, ranging from Europe (Hardy 1938) to China (Liu *et al.* 2000).

The life cycle of DBM is rather short with a developmental time from egg to adult of 14 days at 25°C. Adult females lay up to 300 yellow or green-pale eggs which have an oval, flattened shape. After hatching, larvae feed as leaf miners within the plant tissue and after the larvae have grown, they feed on the external portion of leaves and buds (Figure 1.1). The pupation takes place in a lacy cocoon attached to the leaf of the host plant. Moths overwinter in debris of collards, cauliflower, cabbage, or related crops. Adult moths have a wingspan of about 15 mm and a body length of 6 mm. When the moth is at rest, the distinct diamond-sharping along the back is visible (Figure 1.1).

The foliar tissue except for the leaf veins may be removed completely as a direct result of feeding activities of caterpillars if the larvae infestation rate is high. Thus, this species causes high crop losses, which can reach up to 100 % when left uncontrolled (Calderon and Hare 1986). In large agricultural production areas such as Brazil, the moth is responsible for the greatest losses in several Brassicaceae, particularly during hot and dry seasons (da Silva *et al.* 2012). In Southeast Asia outbreaks of DBM cause sometimes crop losses of more than 90 % (Verkerk and Wright 1996) and in the United States it is one of the major pests of crucifers in various regions (Harcourt 1957, Buntin 1990, Brown *et al.* 1999). In the past years different insecticides including organophosphates, pyrethroids, carbamates and diamides have been used to control DBM successfully. Additionally, natural enemies such as parasitoids of eggs, larvae or pupae are employed (for review see Sarfraz *et al.* 2005). However, parasitoids do not exert adequate control, as they mostly require frequent mass releases (Talekar and Shelton 1993) and egg parasitoids, for example, are not always host-specific thus they can be harmful for non-target species in a region (Goulet and Huber 1993). According to Talekar and Shelton (1993) the annual costs of managing this pest globally is estimated to be one billion \$US in addition to crop loss.

Figure 1.1. Diamondback moth (*P. xylostella*). Left: adult moth. Right: larva feeding on cabbage. Pictures taken by Tibor Bukovinszky, Bayer AG Division Crop Science (www.imagebank.cropscience.bayer.com).

Due to year-round cultivation, frequent insecticide applications are required throughout the growing season in tropical and subtropical areas where problems with DBM reach a great extent (Ribeiro *et al.* 2014). By relying exclusively on chemical control measures, insecticide resistance has been growing and the highest levels of resistance were generally associated with areas of intensive brassica cultivation (Cheng 1986, Tabashnik *et al.* 1987). DBM has a high potential to develop resistance and it has been reported that 95 different insecticides of more than ten mode of action classes with tendencies rising show no effect on this insect.

Apart from strong insecticide selection pressure to which DBM has been subjected, its genetic plasticity, high fecundity and short life cycle in tropical regions are contributing factors that allow it to overcome literally any chemical measure (Oliveira *et al.* 2011, Santos *et al.* 2011).

1.1.2 Tomato leaf miner, *Tuta absoluta*

The tomato leaf miner or tomato borer, *T. absoluta*, is an invasive pest of tomato crops and of global significance due to its destructive feeding activities on different solanaceous plants. Its distribution was restricted to South America but within a few years after invading Europe in 2006, it has rapidly become a worldwide threat for tomato production in both open field and greenhouse crops (Desneux *et al.* 2010, Desneux *et al.* 2011).

The total development from egg to adult of *T. absoluta* is completed in an average of 24-40 days between 27 °C and 20 °C, respectively with 10-12 generations per year depending on weather conditions. Females deposit up to 300 small eggs which are creamy yellow in colour and have a cylindrical shape. After hatching, the larvae bore between the epidermal layers of the leaf forming irregular leaf mines (Figure 1.2). At the stage of third instar larvae leave these mines and infest new locations which include the apical stem and fruits where they mine again. The pupation site varies and pupae can be found in the mines, outside the mine, or in the soil. The adults have a silvery brown colour and are fairly small with a wing span of 10 mm and a body length of approximately 6 mm (Figure 1.2). Overwintering takes place as eggs, pupae or adults depending on environmental conditions.

The primary host of *T. absoluta* is tomato but various solanaceous plants, like eggplant, potato and *physalis*, are suitable as well. The rate of infestation is mainly dependent on the plant variety and when numbers of larvae are high the plant foliage can be destroyed completely. Feeding damage is caused in the whole plant throughout the entire crop cycle. In fruits, the feeding activity of larvae can lead to fruit rot, as the entry wholes serve as open passages for secondary pathogens. The loss in fruit production caused by *T. absoluta* ranges from 50 % to 100 % if not managed properly.

However, the management of this pest can be difficult (for review see Guedes and Picanço 2012), as natural predators such as pirate bugs are not available in areas which have been invaded recently. Consequently, the conventional approach with chemicals remains as the major pest control tactic despite the intensive search for alternatives. Insecticide application with a broad-spectrum activity such as organophosphates, carbamates and pyrethroids are most commonly used on a large scale with up to 30 applications per cultivation period. Thus, in tomato production the costs have more than tripled since the introduction of *T. absoluta* and the concerns for the environment and human safety regarding the frequent insecticide application are rising. Due to its high potential to develop resistance, *T. absoluta* has been reported to be resistant against many classes of insecticides including pyrethroids (Haddi *et al.* 2012),

avermectins (Siqueira *et al.* 2001), spinosyns (Campos *et al.* 2014) and diamides (Roditakis *et al.* 2015).

Figure 1.2. Tomato leaf miner (*T. absoluta*). Left: adult moth, right: larvae. Pictures taken by Dr. Sascha Eilmus, Bayer AG Division Crop Science (www.imagebank.cropscience.bayer.com).

1.2 Insecticides

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Own contribution: 55 %

The discovery, development and registration of novel chemical classes of insecticides with new modes of action, i.e. addressing a yet unexploited/underutilized target protein, or at least interfering with a new binding site on an established insecticide target, are major challenges in modern crop protection research. A challenge, which is – after consolidation of the agrochemical industry – pursued by a rather limited number of R&D based companies, particularly because of high budget needs for insecticide development and registration, often easily exceeding $200 million (Sparks 2013). Major drivers for the discovery and development of new chemical classes of insecticides are an increasing requirement for compounds with improved environmental and toxicological profiles, as well as the global spread of pest

resistance compromising field efficacy of established insecticides and thus directly influencing yield and food supply. A recent survey revealed that in 2013 approximately 70 % of the global insecticide market was based on 5 out of about 55 different chemical classes listed in the insecticide mode of action classification scheme of the Insecticide Resistance Action Committee (IRAC), including neonicotinoids acting on nicotinic acetylcholine receptors (27 % market share), pyrethroids acting on voltage-gated sodium channels (16 %), organophosphates inhibiting acetylcholinesterase (11 %), diamides acting on ryanodine receptors (8 %), benzoylphenyl urea (< 2 %) and avermectins acting on ligand-gated chloride channels (7 %) (Sparks and Nauen 2015). Out of these chemical classes, diamide insecticides represent the most recent class of chemistry introduced to the market approximately 10 years ago (Nauen 2006, Jeanguenat 2013). Whereas pyrethroids and benzoylphenyl urea are a fairly old group and have been on the market for more than 30 years.

1.2.1 Diamide Insecticides and their Mode of Action

1.2.1.1 Diamide Insecticides

Three diamide insecticides, i.e. the benzenedicarboxamide (or phthalic diamide), flubendiamide (Tohnishi *et al.* 2005, Hirooka *et al.* 2007, Hamaguchi and Hirooka 2012) and anthranilic diamides chlorantraniliprole and cyantraniliprole (Lahm *et al.* 2005, 2007, 2009), have so far been commercialised with a global turnover of >$1.2 billion representing approx. 8 % of the insecticide market in 2013 (Sparks and Nauen 2015). However, at least three more diamide insecticides, i.e. cyclaniliprole, tetrachlorantraniliprole and tetraniliprole, are currently under development and expected to be launched to the market within the next few years (Figure 1.3), whilst other, more recently described chemical derivatives such as diamide sulfoximines have not yet revealed development candidates (Gnamm *et al.* 2012). The discovery and development of diamide insecticides has been recently reviewed by Jeanguenat (2013). Whereas flubendiamide and chlorantraniliprole are particularly active at low application rates against a broad range of lepidopteran and lepidopteran/coleopteran pests, respectively, cyantraniliprole – due to its systemic properties – also targets a number of sucking pests including aphids and whiteflies (Foster *et al.* 2012, Li *et al.* 2012, Gravalos *et al.* 2015). However, chlorantraniliprole also exhibits root-systemic properties and can therefore be used by systemic application but mainly against foliar-feeding lepidopteran pests (Cameron *et al.* 2015). Diamide insecticides show low acute mammalian toxicity and a favourable environmental profile and are safe to beneficial insects and mites in many agricultural and horticultural settings investigated. When introduced to the market, diamides did not show any cross-resistance to existing chemical classes, as one would expect for a new chemical class of insecticides addressing a new binding

site (mode of action) on a rather neglected molecular target, the insect ryanodine receptor (RyR).
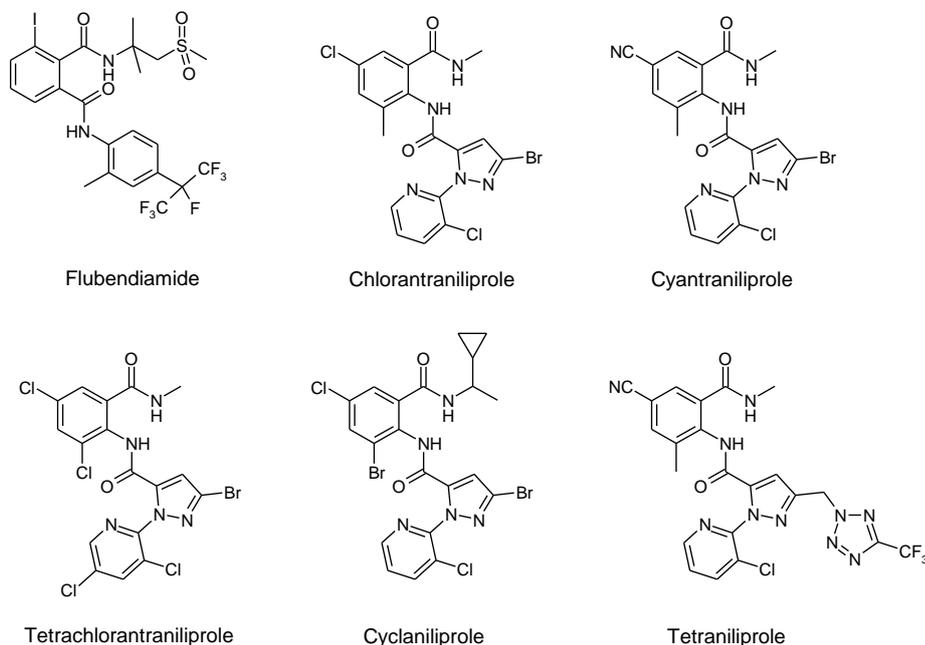
Figure 1.3. Diamide insecticides acting as conformation sensitive activators on insect ryanodine receptors. Flubendiamide (Nihon Nohyaku/Bayer), chlorantraniliprole and cyantraniliprole (DuPont) were launched in 2006, 2007 and 2012, respectively. Tetrachlorantraniliprole (Sinochem), cyclaniliprole (Ishihara) and Tetraniliprole (Bayer) (ISO-proposed common names) are currently under development (Nauen and Steinbach 2016).

1.2.1.2 Ryanodine Receptor and Diamide Mode of Action

Diamide insecticides were shown to act as conformation-sensitive activators of the insect ryanodine receptor (RyR), a large (homo)tetrameric calcium-channel located in the sarco- and endoplasmic reticulum in neuromuscular tissues (Ebbinghaus-Kintscher *et al.* 2006, Cordova *et al.* 2006, 2007, Lümmen *et al.* 2007, Sattelle *et al.* 2008). RyRs are endogenously activated by calcium influx, mediated by voltagegated calcium channels upon depolarization of the cell membrane (Lümmen 2013). By addressing a new binding site of the RyR, diamides cause a calcium-dependent calcium release resulting in the depletion of internal calcium stores which leads to uncontrolled muscle contraction, paralysis and eventually death as shown in lepidopteran larvae (Tohnishi *et al.* 2005, Cordova *et al.* 2006). Due to their new biochemical mode of action (MoA), diamide insecticides were classified by IRAC as ryanodine receptor modulators and assigned to a new main MoA group 28 (Nauen 2006). Whereas mammals possess three RyR isoforms localised in different tissues (Rossi and Sorrentino 2002), insects encode a single RyR gene with an open reading frame of >15,000 nucleotides translated into a

protomer with a molecular weight of more than 5,000 kDa, as first described for *Drosophila melanogaster* (Takeshima *et al.* 1994). These protomers assemble to homotetrameric membrane proteins of >2 MDa forming the largest known ion channels (Hamilton 2005). RyRs were shown to be composed of six helical transmembrane spanning domains at the C-terminal end containing the calcium ion-conducting pore and a large N-terminal cytosolic domain (Lümmen 2013). A mammalian RyR1 structure determined by single-particle electron cryomicroscopy was recently published and provided interesting insights regarding its structural features as it resolves in total 70 % of 2.2 MDa molecular mass homotetrameric channel protein (Yan *et al.* 2015). The RyR as an insecticide target-site has been utilised for decades and is named after the alkaloid insecticide ryanodine isolated from the South American plant species *Ryania speciosa*, known for its insecticidal properties for almost 200 years (Pepper and Carruth 1945, Rogers *et al.* 1948). A major problem of using ryanodine as an insecticide is its toxicity to both insects and mammals due to a lack of selective binding to RyRs (Lehmberg and Casida 1994); however, the synthesis of more selective and potent derivatives largely failed for various reasons (Waterhouse *et al.* 1987). The insecticidal properties of ryanodine were, however, rather limited under field conditions. Earlier work on both natural *Ryania* alkaloids and their semi-synthetic derivatives in order to increase their efficacy – including extensive structure activity relationship studies – failed to exploit this target to produce economically relevant insecticides (Jefferies *et al.* 1997, and references cited therein). Despite its limitations as an insecticide, ryanodine became a unique tool in the characterisation of RyRs owing to its binding specificity and high affinity for insect and mammalian receptors (K_D 5–15 nM). However, diamide insecticides address a different binding site on insect RyRs and act as positive allosteric activators as demonstrated by the increase of [^{3}H]ryanodine binding as a function of diamide concentration with an EC50 value in the nanomolar range to both insect thoracic microsomal membrane preparations as well as functionally expressed RyRs in insect cell lines (Ebbinghaus-Kintscher *et al.* 2006, Lümmen *et al.* 2007, Qi and Casida 2013, Steinbach *et al.* 2015, Troczka *et al.* 2015). Whereas diamides do virtually not bind to mammalian RyR isoforms (Ebbinghaus-Kintscher *et al.* 2006, Lahm *et al.* 2007), they show some species differences in terms of selectivity among insects of different orders (Qi and Casida 2013, Qi *et al.* 2014).
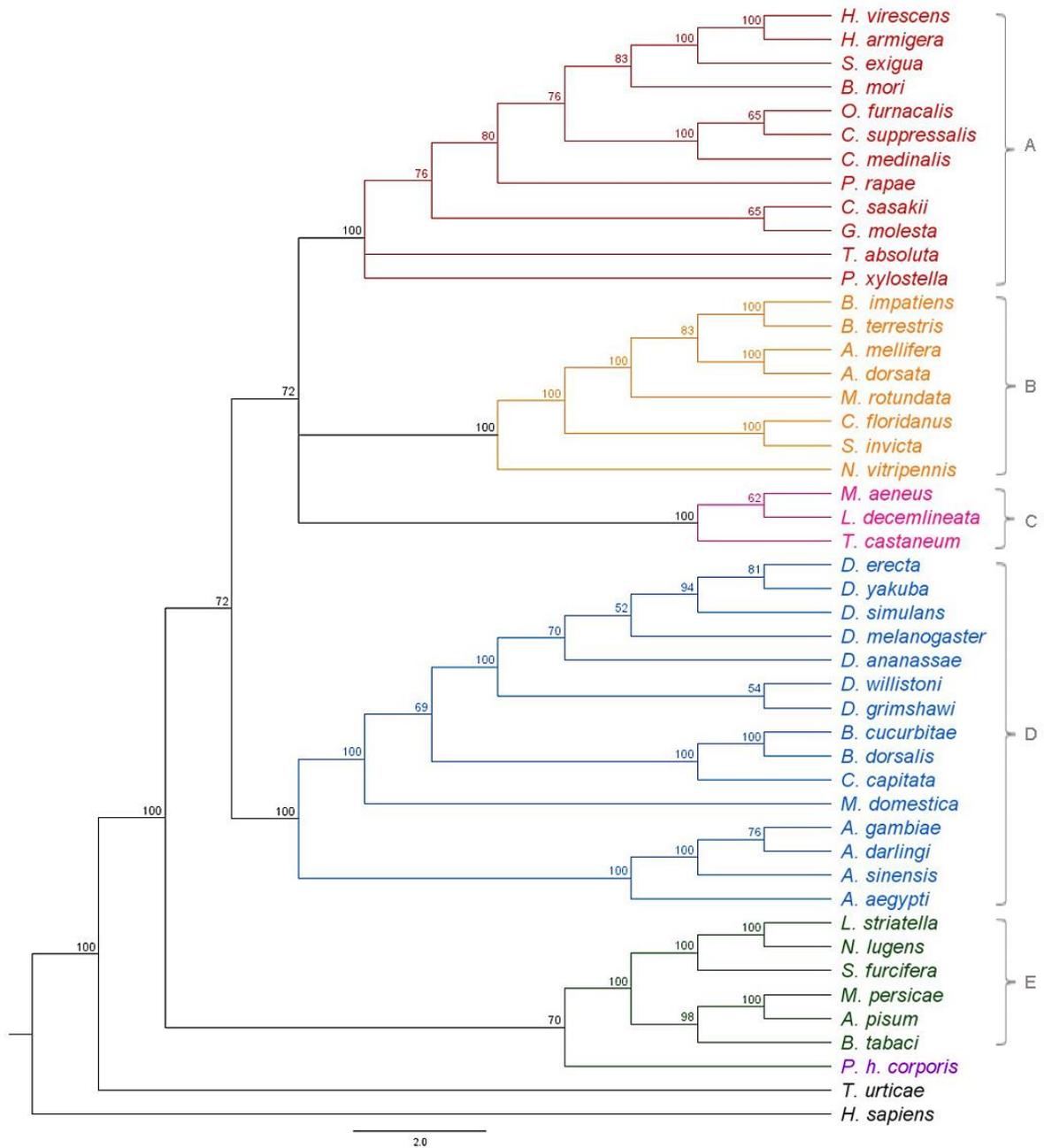
Figure 1.4 Neighbour-joining phylogenetic analysis of the ryanodine receptor (*RyR*) of different insect orders and noninsect species. (**A**) Lepidoptera, (**B**) Hymenoptera, (**C**) Coleoptera, (**D**) Diptera, (**E**) Hemiptera. Root: *Homo sapiens*. The corresponding GenBank accession numbers are as follows: Coleoptera (*Leptinotarsa decemlineata*, AHW99830; *Meligethes aeneus*, unpublished (Nauen *et al.*); *Tribolium castaneum*, AIU40166.1); Diptera (*Aedes aegypti*, Q17EB5; *Anopheles darlingi*, W5JDV8; *Anopheles gambiae*, Q7PMK5; *Anopheles sinensis*, A0A084WAS3; *Bactrocera dorsalis*, A0A034W289; *Bactrocera cucurbitae*, A0A0A1WHX3; *Ceratitis capitata*, W8AL79; *Drosophila ananassae*, XP_001958793.1; *Drosophila erecta*, XP_001970412.1; *Drosophila grimshawi*, XP_001995333.1; *Drosophila melanogaster*, AFH07966.1.

When utilising a photoreactive derivative of flubendiamide against a series of *Bombyx mori* RyR deletion mutants recombinantly expressed in HEK293 cells, Kato *et al.* (2009) concluded that the diamide binding site is likely to be located in the C-terminal transmembrane spanning

domain, which was confirmed by studies on diamide-resistant diamondback moth strains carrying a target-site mutation in the transmembrane domain (Troczka *et al.* 2012, Guo *et al.* 2014a, b, Steinbach *et al.* 2015). Further evidence for a critical role of this transmembrane region for diamide binding was provided by a study replacing a 46 amino acid segment in the *Drosophila* RyR C-terminal domain by that of a nematode RyR which resulted in insensitivity to diamides (Tao *et al.* 2013). Since the introduction of diamide insecticides, several more insect RyR genes were cloned, sequenced and compared by phylogenetic means (Fig. 1.4), including those from lepidopteran pests such as diamondback moth (Wang and Wu 2012), which subsequently allows to investigate the implications of amino acid substitutions for diamide insecticide target-site resistance first described in diamondback moth (Troczka *et al.* 2012, Steinbach *et al.* 2015).

Figure 1.4 (continued) *Drosophila simulans*, XP_002080659.1; *Drosophila willistoni*, XP_002061506.1; *Drosophila yakuba*, XP_002089690.1; *Musca domestica*, XP_011296554.1); Hemiptera (*Bemisia tabaci*, I3VR33; *Laodelphax striatellus*, A0A059XRL5; *Myzus persicae*, A0A0A7RS32; *Nilaparvata lugens*, KF306296; *Sogatella furcifera*, KF734669); Hymenoptera (*Apis mellifera*, AFJ66977.1; *Apis dorsata*, XP_006622367.1; *Bombus impatiens*, XP_012250208.1; *Bombus terrestris*, XP_012175583.1; *Camponotus floridanus*, XP_011257849.1; *Megachile rotundata*, XP_003701507.1; *Nasonia vitripennis*, XP_008202582.1; *Solenopsis invicta*, XP_011158883.1); Lepidoptera (*Bombyx mori*, XP_004924916.1; *Carposina sasakii*, X2GG79; *Chilo suppressalis*, I3VR34; *Cnaphalocrocis medinalis*, I1XB02; *Grapholita molesta*, A0A089FYX0; *Helicoverpa armigera*, V5RE97; *Heliothis virescens*, DD408555.1; *Ostrinia furnacalis*, M4T4G3; *Pieris rapae*, R9R5D5; *Plutella xylostella*, AEI91094.1; *Spodoptera exigua*, A0A059XRP6; *Tuta absoluta*, unpublished data);Vertebrata (RyR 1) (*Rattus norvegicus*, F1LMY4; *Homo sapien*s, P21817; *Oryctolagus cuniculus*, P11716); others (*Pediculus humanus corporis*, E0VEK3; *Tetranychus urticae*, F5HSW9). The phylogenetic tree was generated using tree builder (Geneious 8.0) with 100 bootstrap replications. The scale bar represents 2.0 amino acid substitutions per site.

1.2.2 Benzoylphenyl urea and their Mode of Action

1.2.2.1 Benzoylphenyl urea (BPU)

Insect growth regulators (IGRs), such as chitin synthesis inhibitors (CSIs), are compounds that selectively target arthropod pests as physiologically related processes or target-sites are not present in vertebrates. Chitin synthesis inhibitors interfere with chitin biosynthesis in insects and therefore, are considered safe for most non-(arthropod)target organisms (Doucet and Retnakaran 2012). The Benzoylphenyl ureas (BPUs, IRAC group 15), a subclass of acylureas, are commonly known to inhibit chitin biosynthesis. After more than four decades, roughly 10000 benzoylurea derivatives have been synthesized, and 15 BPU CSIs have been commercialized (Figure 1.5). The discovery and development of the BPUs has recently been reviewed by Sun *et al.* (2015).

The discovery of the BPU dates back to the 1970´s where Dutch scientists attempted to synthesize a novel herbicide by combining two molecules—dichlobenil and diuron at Philips-Duphar B.V. Co. (Van Daalen *et al.* 1972). Surprisingly, the new compound exhibited no herbicidal activity but insecticidal properties, such as larvicidal activities against caterpillars and mosquitos. Following research, the first commercial product was diflubenzuron (1) (Dimilin®), which was launched to the market in 1975 by Philips-Duphar B.V.. The most successful BPU compound after diflubenzuron is triflumuron (6) which was discovered by Bayer AG and introduced to the market in 1979 (Sun *et al.* 2015). Furthermore, it was the first commercial product of the second generation BPUs. Comparing it to the first generation products it showed higher ovicidal as well as broad-spectrum larvicidal activity, especially against lepidopteran pests. The third generation BPUs, such as flufenoxuron (12), exhibited stronger topical insecticidal activity and broader spectrum larvicidal activity against Lepidoptera, Homoptera, Diptera, and Hemiptera pests (Anderson *et al.* 1986, Perugia *et al.* 1986). In summary, the target selectivity is variable across the BPUs and each compound shows different activity against pest species.

In 2013, the BPUs had a market share of U.S. $441 million which accounted for 3 % in the total global market for insecticides (Sparks and Nauen 2015). Despite their small market share, BPUs are still widely used, especially as green insecticides in agricultural crop protection, forestry and sanitary insect pest control against flies and mosquitos (Tomlin 2003). Owing to their low toxicity to mammals (non-neurotoxic MoA) and predatory insects (low contact activity), BPUs play an important role in integrated pest management (IPM) and insecticide resistance management (IRM) programs.
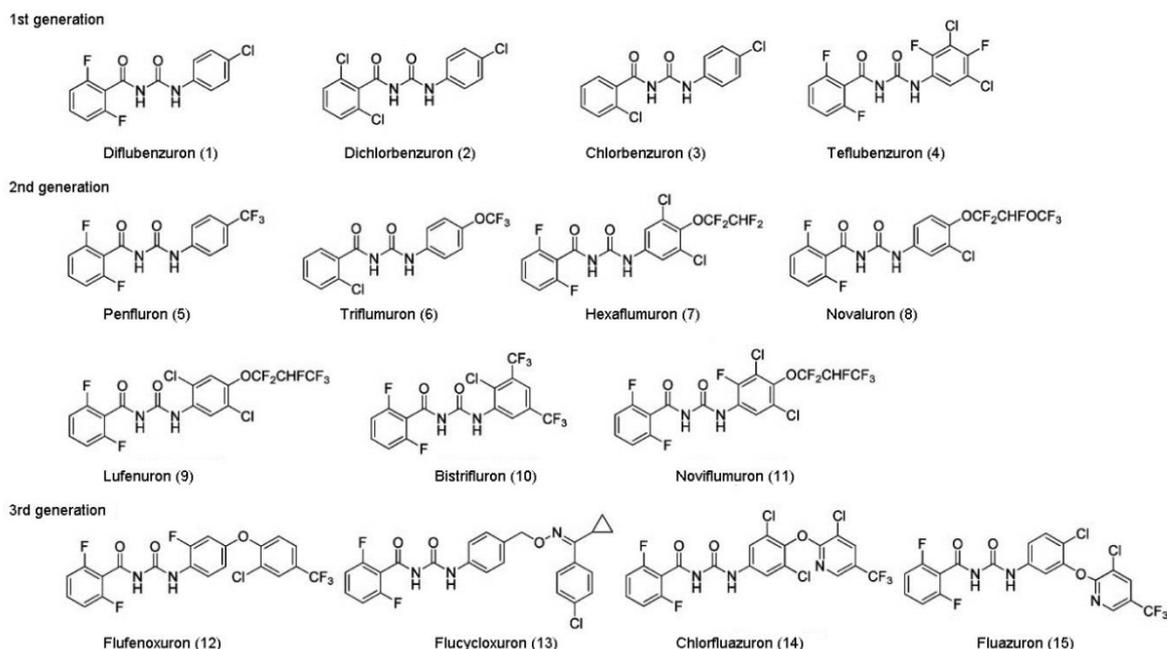
Figure 1.5. Chemical structures of commercial benzoylphenyl urea (BPU) chitin synthesis inhibitors. (1) diflubenzuron (1975, Philip-Duphar B.V., now Platform Specialty Products Co.), (2) dichlorbenzuron (1975, Philip-Duphar B.V., now Platform Specialty Products Co.), (3) chlorbenzuron (1976, JiangSu Institute of Ecomoes Co. Ltd., China), (4) teflubenzuron (1984, Celamerck GmbH & Co. KG, now BASF AG), (5) penfluron (1977, Thompson-Hayward Chemical Co., now Harcros Chemicals, Inc.), (6) triflumuron (1979, Bayer AG), (7) hexaflumuron (1987, Dow AgroSciences), (8) novaluron (1990, Makhteshim-Agan), (9) lufenuron (1990, Ciba-Geigy, now Novartis), (10) bistrifluron (2000, Dongbu Hannong Chemical), (11) noviflumuron (2001, Dow AgroSciences), (12) flufenoxuron (1987, Shell Research Ltd., now BASF AG), (13) flucycloxuron (1988, Philip-Duphar B.V., now Platform Specialty Products Co.), (14) chlorfluazuron (1988, Ishihara Sangyo Kaisha Ltd.), (15) fluazuron (1990, Ciba-Geigy AG, now Novartis).

1.2.2.2 BPU Mode of Action

Chitin, a polymer of *N*-acetyl-β-D-glucosamine, is an essential biopolymer in nature and it is mainly produced by arthropods, fungi and nematodes. The exoskeleton which is formed by the cuticle of insects consists of chitin and sclerotized proteins. This more or less rigid structure limits insect body growth and implicates a periodical replacement of the old cuticle with a new one during molting (ecdysis). As the growth and development of insects depend on the ability to remodel chitinous structures, chitin synthesis is an attractive target for combating insect pests. However, chitin formation and deposition is a complex process (for review see Merzendorfer and Zimoch 2003) and only the polymerization events associated with the cell membrane compartment are so far available for chemical interference (Cohen 1993).

It is known that BPUs interfere with chitin deposition during cuticle formation which leads to an molting defects and inhibition of egg hatching. Furthermore, BPUs cause malformations of the

cuticle, significant reduction of chitin amounts (van Eck 1979, Merzendorfer *et al.* 2012) and prevent the normal formation of the peritrophic membrane (Clarke *et al.* 1977). However, the exact mechanism of action has remained elusive for almost 40 years (for an in depth review on the research concerning the MoA of BPUs see Oberlander and Silhacek 1998 and Matsumura 2010).

Several studies on whole organisms and organ cultures provided evidence that BPUs inhibit the polymerization step during chitin formation (Hajjar and Casida 1978, Mitsui *et al.* 1985) without directly interfering with chitin synthase, the enzyme catalysing the last step during chitin synthesis (Cohen and Casida 1980, Mayer *et al.* 1980, Cohen 1985). On the contrary, other CSIs such as the substrate mimic fungicides polyoxin-D and nikkomyzin-Z were shown to directly inhibit the chitin synthase *in vitro*. Mitsui *et al.* (1985) proposed that the mode of action of diflubenzuron includes inhibition of UDP-*N*-acetyl-D-glucosamine transport across membranes. Additionally, Nakagawa *et al.* (1993) demonstrated that the incorporation of ^{3}H-*N*-acetyl-D-glucosamine was completely suppressed by diflubenzuron in isolated integuments of newly molted American cockroach (*Periplaneta americana*). In contrast to this, Abo-Elghar *et al.* (2004) suggested that the ABC transporter, sulfonylurea receptor (SUR), is the direct target of the BPUs and thus inhibiting chitin biosynthesis indirectly by altering vesicle trafficking. In the latter study diflubenzuron and glibenclamide (sulfonylurea), a known SUR-binding inhibitor, were applied to isolated integuments of the German cockroach, *Blatella germanica* and the inhibitory activity on chitin synthesis of glibenclamide was comparable to diflubenzuron. Furthermore, competitive binding assays revealed that diflubenzuron was able to competitively displace radiolabelled [^{3}H]-glibenclamide. However, the hypothesis of SUR as BPU target-site remained controversial as the direct evidence was missing (Akasaka *et al.* 2006, Gangishetti *et al.* 2009). It was presumed that the effect of BPU and sulfonylurea on chitin synthesis may be similarly indirect (Merzendorfer 2006). Meyer *et al.* (2013) have later shown that the SUR is dispensable for chitin synthesis in *Drosophila melanogaster* by creating a mutant that completely lacked of SUR. These mutants were not lethal and developed a normal chitinous pro-cuticle of wild-type texture and thickness.

After more than 40 years of research, Douris, Steinbach *et al.* (2016) have resolved the molecular MoA of the BPUs by a multistep approach using classic genetics and genome-editing. They uncovered a mutation (I1042M) in chitin synthase 1 (CHS1) of a BPU-resistant *P. xylostella* strain and the frequency of this mutation was highly correlated with cross-resistance to several BPUs including diflubenzuron and triflumuron. Interestingly, this mutation was located at the same position as the mutation I1017F which was identified in the orthologous gene of the spider mite, *Tetranychus urticae*, conferring resistance to the acaricide etoxazole (van Leeuwen *et al.* 2012). In this study, it was demonstrated that the chitin synthase is the target of etoxazole. Earlier studies on the MoA of etoxazole have shown that etoxazole induced

moulting defects in fall armyworm, *Spodoptera frugiperda* larvae identical to those caused by BPUs (Nauen and Smagghe 2006). In order to prove the link between the mutations I1041M and I1017F in *CHS1* and BPU resistance, the two mutations were introduced in *D. melanogaster kkv* (*CHS1* orthologue) by a CRISPR/Cas9 coupled with HDR genome modification approach (Douris, Steinbach *et al.* 2016). The homozygous lines carrying either of these mutations have shown a highly resistant phenotype against etoxazole and all tested BPUs, as well as buprofezin — an important hemipteran chitin biosynthesis inhibitor. This provides compelling evidence that BPUs, etoxazole, and buprofezin share in fact the same molecular MoA and act directly on chitin synthase (Douris, Steinbach *et al.* 2016).

1.3 Insecticide Resistance

Resistance has been defined as "the inherited ability of a strain of some organism to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species" (WHO, 1957).

The problem of insecticide resistance is geographically widespread and occurs in at least 515 insect species (http://www.pesticideresistance.org/, Feb. 2017) according to current knowledge. Therefore, factors that govern the origin and spread of resistance-associated mutations are both of academic and of applied importance (ffrench-Constant *et al.* 2004).

The speed by which resistance arises and spreads is highly dependent on the type of crop protection product as well as its target specificity, its timing of application during the generation time of the pest and its application rate (Russel 2001). In addition to this agronomic risk factor, the inherent risk concerning the reproduction capacity, life cycle and migration of the pest is crucial. The issue of cross-resistance makes pest control even harder as this phenomenon enables the insect to overcome the toxicity of another insecticide that shares the same MoA even when the insect has never been exposed to the insecticide.

In general, the nature of resistance can be monogenic, where single alleles confer high tolerances against insecticides (ffrench-Constant *et al.* 1998) or polygenic/multigenic where more than one resistance mechanism is enforced (Groeters and Tabashnik 2000). Thus, the evolution of high resistance levels is triggered by frequent uses of insecticides by providing a strong selective force in a pest population (Hardstone and Scott 2010). The genetic changes that are employed are diverse and comprise: crossing over events (Joußen *et al.* 2012), gene amplification (Bass and Field 2011), DNA methylation (Field *et al.* 1996), single nucleotide polymorphisms (SNPs) (Troczka *et al.* 2012), regulatory tandem repeats (Bass *et al.* 2013), transposable elements (Wilson 1993), RNA editing (Dong 2007, Es-Salah *et al.* 2008) and alternative and/or mis-splicing (Sonoda *et al.* 2006, Xiao *et al.* 2014). Alterations in DNA/RNA lead to complex changes in insect physiology and they can be further categorized into four resistance mechanisms that will be exemplified in the next sections.

1.3.1 Mechanisms of Insecticide Resistance

In general, there are four main mechanisms which can increase the insensitivity of insects to the insecticide and they are classified as: 1) penetration resistance, 2) behaviour resistance, 3) metabolic resistance and 4) target-site resistance.

Penetration resistance is characterized by a much slower absorption of the toxin through the body wall of resistant individuals in comparison to the susceptible counterpart, e.g. by changes in cuticle structure such as enhanced thickness of the cuticle (Ahmad and McCaffery 1999). However, this mechanism is considered not efficient enough on its own and therefore, only a contributing factor to insecticide resistance. This is similar to behavioural resistance, resulting for example in contact avoidance of insecticide baits as shown in cockroaches (Wada-Katsumata *et al.* 2013)

Target-site resistance or metabolic resistance alone or the presence of both mechanisms in combination, confer high levels of resistance to entire chemical classes of insecticides. Therefore, they are explained in detail in the next two sections down below.

1.3.2 Metabolic Resistance

The metabolism of insecticides is a biochemical process whereby insecticides are broken down into non-toxic forms (Perry *et al.* 2011). The breakdown of insecticides can be a rather complex process involving multiple enzymes. It is, however, a process that occurs in all insects and therefore, it is crucial to distinguish insecticide metabolism from resistance.

In general, the metabolism of xenobiotics can be divided into three phases (Phase I-III). Phase I involves two main enzyme-groups: cytochrome P450 monooxygenases (CYPs) and esterases (ESTs). These enzymes are able to modify chemical compounds thereby introducing hydrophilic functional groups into lipophilic substrates by reactions, such as oxidation, reduction and hydrolysis. The membrane-bound hemoprotein superfamily of P450s function as monooxygenases, whereas cytosolic esterases work as hydrolases. The metabolites of Phase I metabolism are either directly excreted or further modified by Phase II metabolism. GlutathioneS-transferases (GSTs) are key enzymes in Phase II metabolism and convert lipophilic and/or P450 hydroxylated xenobiotics to hydrophilic products by glutathione conjugation. Therefore, GSTs enable the rapid removal of Phase I metabolites from cells and facilitate the excretion. Finally, the conjugated xenobiotics are actively eliminated from cells by ATP binding cassette (ABC) and other major membrane transporters (Phase III).

Metabolic resistance is predominantly characterised by genomic changes that lead to amplification, overexpression, and coding sequence variation in the three major groups of genes encoding for metabolic enzymes (CYPs, GSTs, ESTs) (Li *et al.* 2007, Vontas *et al.* 2001)

thereby allowing the insect to overcome the toxicity of the insecticide. Mutations are one of the key factors involved in the increased levels of insecticide sequestration as they can induce the production of more detoxification enzyme, either by gene amplification or gene duplication events, or enhanced gene transcription (Daborn *et al.* 2002, Field *et al.* 1999, Hemingway 2000, Schmidt *et al.* 2010). Moreover, point mutations in the coding sequence of genes can result in the alteration of kinetics or substrate specificity of detoxification enzymes (Claudianos *et al.* 1999, Newcomb *et al.* 1997). Metabolic resistance is one of the most common types of resistance in insects due to the high diversity of molecular mechanisms that result in the enhancements of insecticide breakdown (Scott 1999).

1.3.3 Target-site Resistance

Target-site resistance (also known as target-site modification or mutation) is caused by a modified target protein structure or abundance of the protein which leads to an insensitivity towards the insecticide. In many cases a substitution of a single amino acid in a target protein was linked to insecticide resistance in pest insects for a broad range of insecticides, including BPUs (Douris, Steinbach *et al.* 2016), organophosphates (Russel *et al.* 2004), neonicotinoids (Liu *et al.* 2005) and diamides (Troczka *et al.* 2012). High resistance ratios are often the results of target-site insensitivity (Tang *et al.* 1997, Schuler *et al.* 1998, Steinbach *et al.* 2015). However, the level of resistance is dependent upon the structural properties of the insecticide which defines its intrinsic activity as some analogous are more likely to bind to the target protein and hence, are more toxic than others (negative cross-resistance). The identity of target-site mutations can be explained by the necessity for resistant targets to maintain their wild-type functions, and therefore a limited number of amino acid replacements can be tolerated in important receptors and enzymes (ffrench-Constant 1999). In general, target-site alterations often exhibit cross-resistance for insecticides that share the same mode of action.

In the case of a monogenic response where a single gene is responsible for the resistance to the respective insecticide the target gene is highly important as the monogenic resistance is in most cases completely stable. In contrast, polygenic resistance which involves multiple genes is rather variable and often influenced by the environment such as frequency of insecticide application.

1.4. Diamide Resistance in Lepidopteran Pests

Owing to their low application rates and high insecticidal efficacy, diamide insecticides were readily used right after their launch in 2006/2007 on a rather extensive scale for the control of several lepidopteran pests, especially in Southeast Asia and China. Meanwhile diamide insecticides are globally used both solo and in mixtures by millions of farmers for foliar, drench

and seed treatment applications in a broad range of agricultural and horticultural cropping systems, thus facilitating the evolution of insect resistance due to increasing selection pressure, particularly on lepidopteran pests (Teixeira and Andaloro 2013). As a result of their frequent use and due to the lack of alternatives of similar efficacy, first cases of diamide field failure were reported only 2 years after launch in the Philippines and Thailand in cabbage against diamondback moth, *P. xylostella* (Troczka *et al.* 2012), a notorious lepidopteran pest in cruciferous vegetables. Subsequently high levels of diamondback moth resistance to diamides compromising the effectiveness of field recommended rates were confirmed in China (Wang and Wu 2012, Wang *et al.* 2013, Gong *et al.* 2014), Brazil (Ribeiro *et al.* 2014), Taiwan, India, USA, Japan, Korea and Vietnam (Steinbach *et al.* 2015). Lepidopteran pests other than diamondback moth which developed high confirmed levels of diamide resistance include tomato leaf miner, *Tuta absoluta* (Roditakis *et al.* 2015), and smaller tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014). Whereas low to moderate resistance ratios in laboratory assays were reported for rice stem borer, *Chilo suppressalis* (Gao *et al.* 2013, He *et al.* 2014); beet armyworm, *Spodoptera exigua* (Lai *et al.* 2011, Che *et al.* 2013); oriental leafworm, *Spodoptera litura* (Su *et al.* 2012, Sang *et al.* 2015); rice leaffolder, *Cnaphalocrocis medinalis* (Zhang *et al.* 2014); soybean looper, *Chrysodeixis includens* (Owen *et al.* 2013); and the obliquebanded leafroller, *Choristoneura rosaceana* (Sial *et al.* 2011, Sial and Brunner 2012). Some lepidopteran pest species are known for their (geographic and intrinsic) variation in response to insecticides, and talking about resistance is misleading in those cases as one has to keep in mind that such variation is to some extent natural and not directly linked to resistance development based on selection pressure or cross resistance issues. Such a variation in response was recently also confirmed in several baseline susceptibility studies with diamide insecticides, including high-risk pests, such as *Helicoverpa armigera* (Bird 2015), *C. suppressalis* (Su *et al.* 2014), *S. litura* (Su *et al.* 2012) and *T. absoluta* (Campos *et al.* 2015).

Table 1.1. Selected studies of either field or laboratory-selected (Lab) resistance to diamide insecticides in Lepidopteran pests.

Species	Common name	Source	Diamide[a]	RR[b]	Mech[c]	Reference
Adoxophyes honmai	Smaller tea tortrix	Field	CPR	77	-	Uchiyama and Ozawa 2014
			FLB	105		
Chilo suppressalis	Striped rice stem borer	Field	CPR	10	-	Gao *et al.* 2013
		Field	CPR	15	M	He *et al.* 2014
		Field	CPR	22	-	Su *et al.* 2014
Choristoneura roseceana	Oblique-banded leafroller	Field	CPR	4	-	Sial *et al.* 2010
		Lab	CPR	8	M	Sial &Brunner 2012
Chrysodeixis includes	Soybean looper	Field	CPR	6	-	Owen *et al.* 2013
			FLB	9		
Cnaphalocrocis medinalis	Rice leaffolder	Field	CPR	9	-	Zhang *et al.* 2014
Plutella xylostella	Diamondback moth	Field	CPR	>1000	T	Troczka *et al.* 2012
			FLB	>1000		
		Field	CPR	>1000	-	Wang and Wu 2012
		Field	CPR	>1000	M/T?	Lin *et al.* 2013
		Lab	CPR	670	M/T?	Wang *et al.* 2013
		Field	CPR	>1000	T	Gong *et al.* 2014
		Field	CPR	>1000	-	Ribeiro *et al.* 2014
		Field	CPR	>1000	T	Guo *et al.* 2014b
		Lab	CPR	48	M	Liu *et al.* 2015
			CYA	3		
			FLB	7		
		Field	CPR	>1000	T	Steinbach *et al.* 2015
			CYA	>1000		
			FLB	>1000		
Spodoptera exigua	Beet armyworm	Field	CPR	164	M?	Lai *et al.* 2011
		Field	CPR	44	-	Che *et al.* 2013
Spodoptera litura	Oriental leafworm	Field	CPR	24	-	Su *et al.* 2012
		Lab	CPR	80	M	Muthusamy *et al.* 2014
		Field	CPR	15	M	Sang *et al.* 2015
			CYA	16		
Tuta absoluta	Tomato leafminer	Field	CPR	>1000	-	Roditakis *et al.* 2015
			FLB	>1000		

[a] Diamide insecticides: CPR, chlorantraniliprole; CYA, cyantraniliprole; FLB, flubendiamide

[b] RR = resistance ratio; highest reported ratio of LC_{50} or LD_{50} of resistant strain/LC_{50} or LD_{50} of susceptible strain.

[c] Mech = mechanism of resistance suggested in the study cited (if known): M = metabolic; T = target-site mutation; - = unknown.

Diamide resistance ratios exceeding 1000-fold were yet only reported in diamondback moth and tomato leaf miner (Table 12.1), suggesting that some insect pests carry a higher potential to develop resistance to diamides than others. Whereas high levels of diamide resistance in diamondback moth is globally on the move as demonstrated by its documented presence in more than ten countries (Steinbach *et al.* 2015), highly resistant tomato leaf miner populations were yet only isolated from vegetable greenhouses in southern Italy (Roditakis *et al.* 2015). The molecular mechanisms conferring diamide resistance in *T. absoluta* are largely unknown and currently under investigation by research groups in Germany, the UK, Greece, Spain and Brazil. Diamondback moth is known as a notorious candidate for rapid resistance development to almost all chemical classes of insecticide introduced for its control, particularly in (sub)tropical areas with intensive use of crop protection products (Talekar and Shelton 1993, Teixeira and Andaloro 2013). For this reason it was not surprising that diamide (cross) resistance was first described in diamondback moth. The underlying mechanisms so far investigated are largely due to target-site mutations in the transmembrane domain of the RyR and not mediated by metabolic mechanisms such as overexpressed detoxification enzymes.

1.4.1 Metabolic Resistance

Phase I metabolism of diamide insecticides in animals depends particularly on microsomal monooxygenases, i.e. cytochrome P450s. It has been reported that flubendiamide metabolism in rats is mainly driven by multistep oxidation of methyl groups (Justus *et al.* 2007), and a major metabolic pathway of chlorantraniliprole and cyantraniliprole in the goat and rat, respectively, was shown to be the hydroxylation of the *N*-methyl and methylphenyl carbons resulting in hydroxyl metabolites (Gaddamidi *et al.* 2011, Yoshida and McGregor 2014). Virtually nothing has been published yet regarding the metabolic fate of diamide insecticides in target organisms such as lepidopteran larvae. Metabolic resistance can be characterised by the genomic changes that lead to amplification, overexpression and coding sequence variation in the three major groups of gene superfamilies encoding for metabolic enzymes such as cytochrome P450s, carboxylesterases and glutathione S-transferases (Li *et al.* 2007), thus allowing the insect to overcome the toxicity of the insecticide. Studies on synergism by co-applying inhibitors of major detoxification mechanisms usually provide a first line of evidence for the presence of metabolic resistance in resistant strains.

However, as major routes of detoxification in animals were shown to include oxidation, it seems appropriate to assume that cytochrome P450-driven metabolisation of diamides in pest insects may potentially mediate metabolic resistance if such enzymes are overexpressed due to prolonged selection pressure. However, even though diamides are used to control lepidopteran pests for almost 10 years, conclusive evidence of metabolic mechanisms of resistance compromising diamide efficacy at recommended field rates was not yet described. Field-

collected strains of those species showing resistance ratios greater than 1000-fold, such as diamondback moth, were shown to express target-site resistance mediated by amino acid substitutions in the transmembrane domain of the RyR (Troczka *et al.* 2012, Guo *et al.* 2014b, Steinbach *et al.* 2015), or, such as tomato leaf miner, no concrete informations on the mechanisms of resistance were reported (Roditakis *et al.* 2015). Campos *et al.* (2015) tested both flubendiamide and anthranilic diamides against a number of field-collected strains of *T. absoluta*, and whilst the level of cytochrome P450 activity was significantly correlated with the variation in chlorantraniliprole and cyantraniliprole susceptibility, no such correlation was evident for the observed variation in flubendiamide efficacy. Though the observed overall variation in lethal concentration values among all tested tomato leaf miner strains against anthranilic diamides was low, it is interesting to note that those with the lowest LC50 values were also those with the lowest cytochrome P450 activity, a fact which suggests that oxidative metabolism determines at least to some extent the observed efficacy variation (Campos *et al.* 2015). The possible involvement of oxidative metabolism in diamide resistance was also suggested in a laboratory-selected Indian strain of *S. litura* exhibiting 80-fold resistance to chlorantraniliprole, but synergist studies using piperonyl butoxide (PBO) were not conclusive both in vitro and in vivo (Muthusamy *et al.* 2014). However, studies on Chinese *S. litura* strains failed to correlate low-level anthranilic diamide resistance with elevated levels of cytochrome P450 activity (Su *et al.* 2012, Sang *et al.* 2015). Another noctuid species investigated for its capacity to develop chlorantraniliprole resistance after several laboratory selection cycles was *S. exigua* (Lai *et al.* 2011). Although elevated levels of cytochrome P450 and esterase activity were measured, their inhibition by synergists did not significantly increase diamide susceptibility in the selected laboratory strain. This is in contrast to diamondback moth where Liu *et al.* (2015a) demonstrated high PBO-mediated synergism of chlorantraniliprole activity in a moderately resistant strain selected for 52 generations under laboratory conditions, suggesting the involvement of increased oxidative metabolism, because the carboxylesterase inhibitor S,S,S-tributyl-phosphorotrithioate (DEF) failed to significantly synergise chlorantraniliprole, thus confirming earlier studies on a field-collected diamondback moth strain (Wang *et al.* 2013). In another study, laboratory selection of cyantraniliprole resistance in diamondback moth resulted in an increased cross-resistance to flubendiamide and chlorantraniliprole and could be synergised to some extent by PBO and diethyl maleate (DEM) (Liu *et al.* 2015b). A recent RNA-seq approach to investigate the transcriptome of three diamondback moth strains exhibiting low, moderate and high levels of chlorantraniliprole resistance revealed a correlation between the level of resistance and the up-regulation of a number of detoxification genes, such as cytochrome P450s, but also downregulation of RyR contigs (Lin *et al.* 2013), a phenomenon also described for other diamide-resistant diamondback moth strains (Gong *et al.* 2014). However, this is in contrast to other studies showing upregulation of RyR transcripts to be

involved in diamide resistance (Yan *et al.* 2014, Liu *et al.* 2015a). Strong synergism of chlorantraniliprole by PBO as well as DEF was recently described in a field-collected strain of a major rice pest, *C. suppressalis*, suggesting a role for both monooxygenases and esterases in the detoxification of chlorantraniliprole (He *et al.* 2014). Interestingly increased esterase activity was also found in a chlorantraniliprole-selected strain of *Choristoneura rosaceana* (Sial *et al.* 2011), and subsequent synergist studies principally supported the role of hydrolytic enzymes in chlorantraniliprole detoxification (Sial and Brunner 2012). In conclusion it seems fair to claim that most if not all studies on lepidopteran pests so far published failed to clearly demonstrate strong implications of metabolic mechanisms of diamide resistance causing field failure at recommended rates, but this may (will) change in the future. However, the growing tendency to utilize technologies such as RNA-seq for transcriptome assembly and expression analysis will for sure facilitate the identification of specific biochemical mechanisms and candidate genes to be principally capable to confer metabolic resistance to diamide insecticides in pest species under continuous selection pressure.

1.4.2 Target-site Resistance

Early studies on the mechanisms of diamide resistance conducted in two diamondback moth strains collected in the Philippines and Thailand revealed an amino acid substitution G4946E in the C-terminal region of the *Plutella* RyR (Troczka *et al.* 2012). The amino acid substitution was shown to have evolved independently in diamondback moth populations in the Philippines and Thailand by different non-synonymous single-nucleotide polymorphisms, i.e. GGG to GAA and GGG to GAG, respectively, both replacing a glycine by a glutamic acid residue. Subsequently other groups confirmed the presence of the G4946E mutation also in diamondback moth populations collected in China (Gong *et al.* 2014, Guo *et al.*2014a, b, Yan *et al.* 2014) and other countries including India, Japan and the USA (Steinbach *et al.* 2015). Some studies also demonstrated that RyR transcript levels are either increased or decreased in addition to the G4946E mutation in diamide resistant strains (Yan *et al.* 2014, Gong *et al.* 2014, Liu *et al.* 2015a). The fact that the G4946E mutation was found in populations from different geographies indicates once more that it evolved independently rather through migration of one population.

The G4946E substitution is located in the RyR transmembrane domain approx. comprising 700 amino acids and suggested as crucial for the binding of diamides in earlier studies conducted with a photoreactive derivative of flubendiamide in RyR deletion mutants of *B. mori*, recombinantly expressed in human embryonic kidney cells (Kato *et al.* 2009). The RyR transmembrane domain is highly conserved among different insect taxa (Figure 1.6), and homology modelling revealed that glycine 4946 is located at the interface between helix S4 and the S4–S5 linker (Steinbach *et al.* 2015), supposed to have a critical role in RyR gating by

impacting the movement of pore-associated helices (Ramachandran *et al.* 2013). Phylogenetic analysis of the RyR of different insect orders reveal that lepidopteran species, which have >90 % homology in their amino acid sequence, share around 78 % homology to Coleoptera and Hymenoptera (Figure 1.4). Other insect RyR isoforms, such as Diptera and Hemiptera, show a 75–77 % identity with Lepidoptera. As shown in Fig. 1.6, the Cterminal transmembrane part of the RyR is a highly conserved region especially in the transmembrane helices, whereas the cytoplasmic part of the protein has diverged during evolution (Lümmen 2013). The G4946E mutation was first described in 2012 and associated with a diamide-resistant phenotype of diamondback moth, but convincing functional evidence for its implications in diamide binding was only provided recently (Steinbach *et al.* 2015). It was shown in radioligand binding studies using thoracic microsomal membrane preparations of diamondback moth that the G4946E mutation has functional implications on both diamide-specific binding as well as on its concentration-dependent allosteric modulation of [3H]ryanodine binding (Steinbach *et al.* 2015). In contrast to thoracic microsomal membrane preparations of a diamide susceptible strain, a diamide-resistant *Plutella* strain did not show specific saturable binding of a tritiated des-methylated flubendiamide analogue, [3H]PAD1. The tritiated diamide radioligand showed nanomolar binding affinities to membrane preparations of susceptible diamondback moth (KD-value 2.7 nM), but no conclusive equilibrium kinetics with membranes isolated from a resistant strain. Thus, Steinbach *et al.* (2015) provided for the first time functional evidence that the G4946E mutation confers RyR target-site resistance to diamide insecticides. The importance of the G4946E mutation for diamide resistance was confirmed in another study using clonal Sf9 cell lines stably expressing either the *Plutella* wild type or G4946E RyR (Troczka *et al.* 2015). It was shown that the binding of both phthalic and anthranilic diamides was dramatically impaired by the G4946E mutation in *Plutella* RyR recombinantly expressed in clonal Sf9 cell lines. Apart from the functional mutation G4946E, three more mutations, E1338D, Q4594L and I4790M, were recently identified in the RyR of a highly resistant *P. xylostella* strain from China and supposed to be involved in diamide resistance (Guo *et al.* 2014b). The critical role of the transmembrane domain at the interface between helix S4 and the S4–S5 linker for diamide binding seems obvious regarding the functional implications of G4946E in diamide binding. Interestingly the mutation site I4790M described by Guo *et al.* (2014b) in the upper helix S2 exhibits a greater diversity among insect taxa, but is located directly opposite of the G4946E mutation as shown in homology models of the diamondback moth RyR based on rabbit RyR1 (Steinbach *et al.* 2015). The distance between the respective C' atom positions of the mutation sites is approx. 13 Å (Figure 1.7). However, functional evidence showing the impairment of diamide insecticide binding by the presence of I4790M, either alone or in combination with G4946E, is still missing. On the other hand, it is tempting to speculate that differences in chlorantraniliprole and flubendiamide binding affinity (and selectivity) recently described in

Musca domestica and *Apis mellifera* membrane preparations (both M4790) in comparison to Lepidoptera (I4790) (Qi and Casida 2013, Qi *et al.* 2014) are based on such less conserved residues rather than G4946. According to the recently published closed-state cryo-EM structure of rabbit RyR1 (Yan *et al.* 2015), the third mutation described by Guo *et al.* (2014b), Q4594L, is not located within the transmembrane domains, but in a region with several predicted EF hand domains (Takeshima *et al.* 1989). The implication of this mutation for diamide binding in lepidopteran RyRs also needs further investigation in the future, similar to E1338D which is located towards the N-terminus of *P. xylostella* RyR. Therefore, it is not in proximity to the other transmembrane-linked mutations (Guo *et al.* 2014b) and the putative binding site of diamide insecticides (Kato *et al.* 2009, Steinbach *et al.* 2015). In summary there is compelling evidence that the substitution of amino acid residue G4946 in RyRs plays a key role in diamide insecticide resistance, albeit its role in other species than diamondback moth yet needs to be explored. On the other hand I4790 is likely to be another important RyR mutation site possibly linked to diamide species specificity (and resistance).
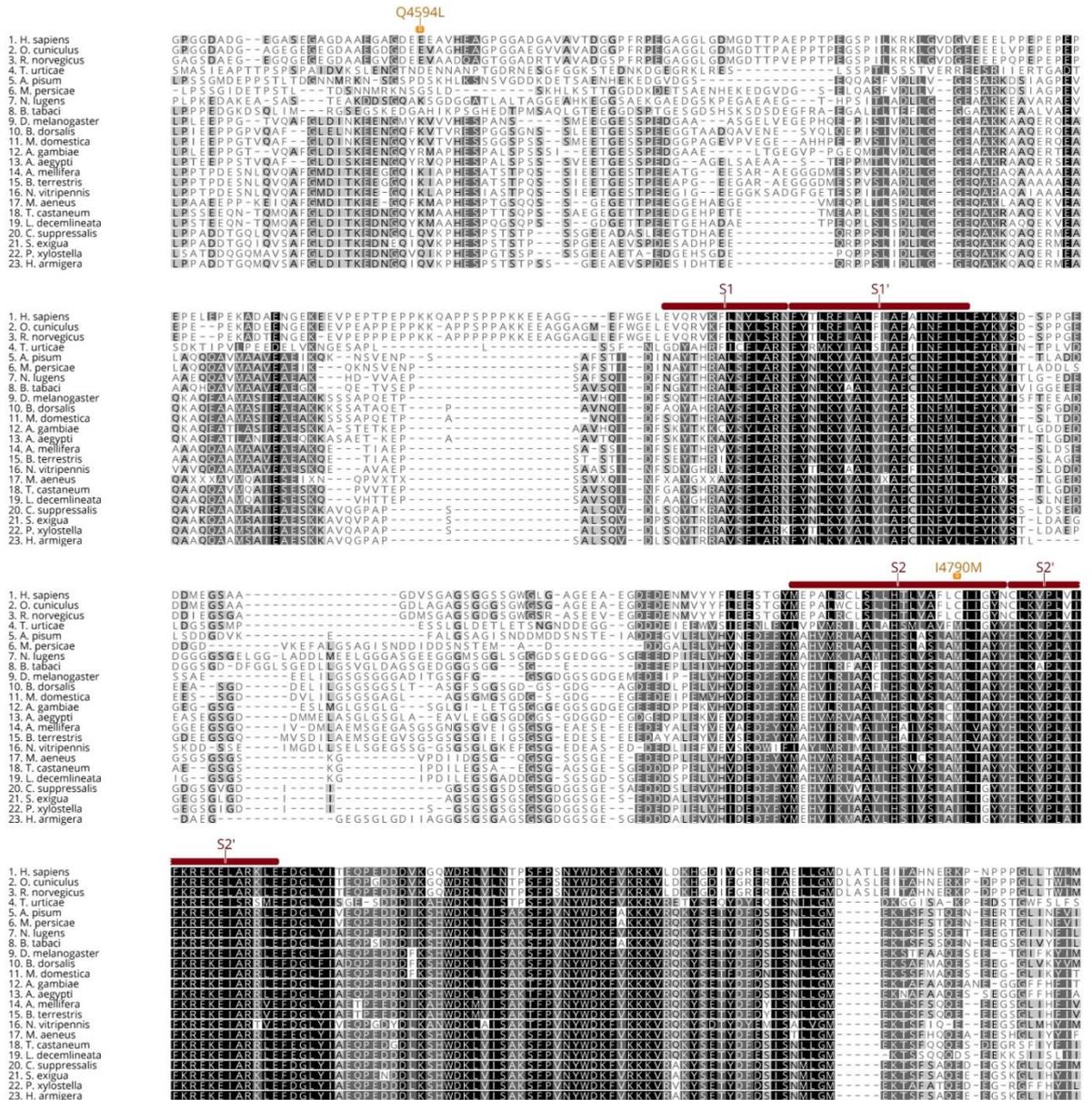
Figure 1.6 Amino acid sequence alignment of the extended C-terminal transmembrane domain of ryanodine receptor (*RyR*) orthologues from mammals and arthropod species covering a broad phylogenetic range. Conserved amino acid residues across species are shaded in *black*. Secondary structural elements and domains are indicated above the alignment by coloured bars and based on a recently published rabbit RyR1 structure (PDB code: 3J8H) determined by single-particle cryomicroscopy (Yan *et al.* 2015). RyR mutation sites linked to diamide insecticide resistance in diamondback moth (*P. xylostella*) are located at positions Q4549L, I4790M and G4946E (numbering based on diamondback moth RyR).
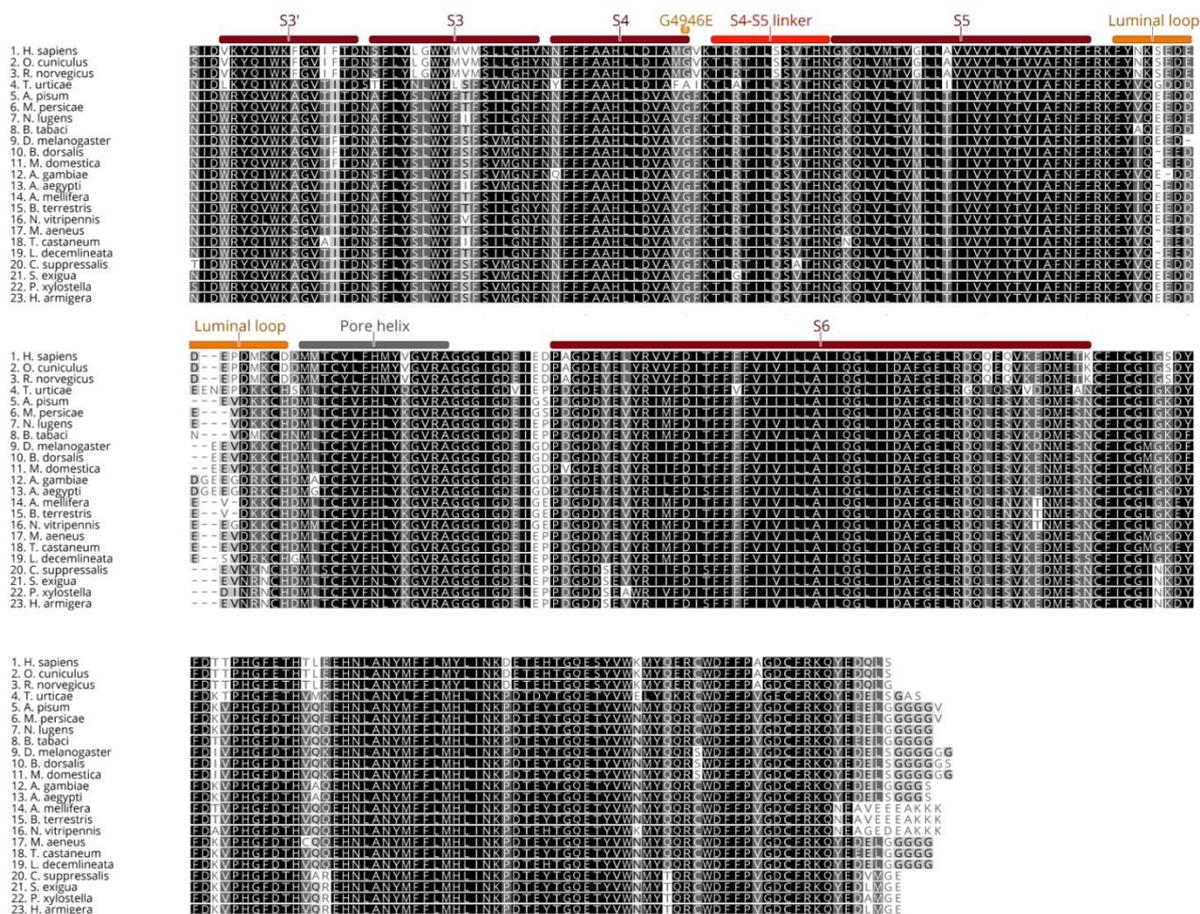
Fig. 1.6 (continued). GenBank accession numbers are as follows: *Homo sapiens*, P21817; *Oryctolagus cuniculus*, P11716; *Rattus norvegicus*, F1LMY4; *Myzus persicae*, A0A0A7RS32; *Nilaparvata lugens*, KF306296; *Bemisia tabaci*, I3VR33; *Drosophila melanogaster*, AFH07966.1; *Bactrocera dorsalis*, A0A034W289; *Musca domestica*, XP_011296554.1; *Anopheles gambiae*, Q7PMK5; *Aedes aegypti*, Q17EB5; *Apis mellifera*, AFJ66977.1; *Bombus terrestris*, XP_012175583.1; *Nasonia vitripennis*, XP_008202582.1; *Meligethes aeneus*, Nauen *et al.* unpublished; *Tribolium castaneum*, AIU40166.1; *Leptinotarsa decemlineata*, AHW99830; *Chilo suppressalis*, I3VR34; *Spodoptera exigua*, A0A059XRP6; *Plutella xylostella*, AEI91094.1; *Helicoverpa armigera*, V5RE97.
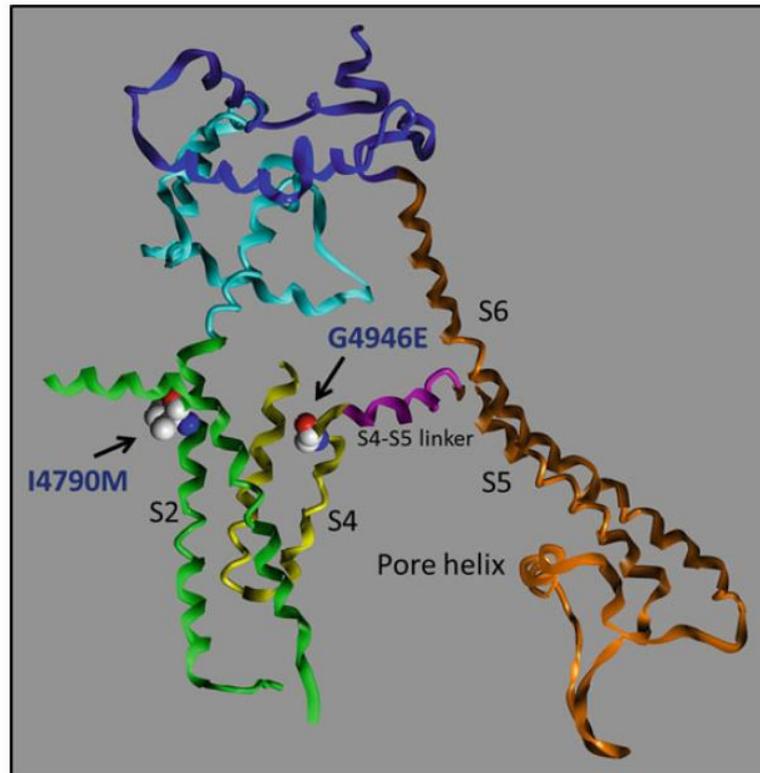
Fig. 1.7 Ryanodine receptor protomer modelling based on the recently published structure of rabbit RyR1 (PDB code 3J8H, Yan *et al.* 2015). Two mutations conferring diamide insecticide resistance in diamondback moth (Troczka *et al.* 2012, Guo *et al.* 2014a), G4946E and I4790M, are located in transmembrane domains S4 and S2 (Steinbach *et al.* 2015).

1.5 BPU Resistance in Lepidopteran Pests

BPUs were massively used with up to 6-8 sprays per crop cycle after their first introduction into the market in the 1970's until the end of the 20th century due to their high pest activity as well as specificity (Syed 1992). Most commonly chlorfluazuron, triflumuron and teflubenzuron were used to control lepidopteran pests, especially DBM. Diflubenzuron however, was not effective against DBM as it had shown moderate resistance against it and therefore, it has never been used for DBM control measures. As a result of the frequent application of BPUs, reports of field failures were accumulating leading to yield losses up to 60 % (Moffit *et al.* 1988) and resistance against BPUs was vastly spreading. The severity of this issue is best shown in Taiwan, where field collected DBM have shown a 243 fold higher tolerance to chlorfluazuron and teflubenzuron only 6 months after their first application in 1987 (Cheng *et al.* 1988, Cheng *et al.* 1990). In other crucifer-growing areas such as Brazil, Thailand, Malaysia, or China similar cases were reported and resistance was found in all of the tested DBM populations not long after BPU introduction (Vattanatangum 1988, Sayed 1992, Yin *et al.* 2011, Xia *et al.* 2014). Interestingly, Malaysian DBM populations which originated from the lowlands were significantly more resistant than those from the highlands due to the shorter generation time of

only 14 days in the lowlands compared to 28 days in the highlands (Sayed 1992). Fahmy and Miyata (1990) were able to select for chlorfluazuron resistance in two field collected DBM from Thailand. Within a relatively short time of 9 and 10 generations, the two strains exhibited a >210 fold greater tolerance to chlorfluazuron. Similar results were obtained when selecting a field collected DBM strain from the Philippines with triflumuron (Douris, Steinbach *et al.* 2016). In this study, the triflumuron concentration was incrementally increased which lead to a stable phenotype that tolerated high doses of triflumuron with resistance ratios >188 fold. Thus, this strain has shown cross-resistance to all other IGRs tested including flucycloxuron (>1000 fold), lufenuron (196 fold) and diflubenzuron (28 fold). However, teflubenzuron resistant codling moth, *C. pomonella*, populations from commercial apple orchards in Czech Republic did not exhibit cross-resistance to diflubenzuron (Stará and Kocourek 2007). In one case a decline in resistance from 7621- and 243-fold to only 6.5- and 3.1-fold for teflubenzuron and chlorfluazuron, respectively, was observed in BPU resistant DBM after 17 selection free generations (Cheng *et al.* 1990).

Several mechanisms have been described for BPU resistance in lepidopteran pests. Studying the metabolism of diflubenzuron and teflubenzuron in fifth instar larvae of beet armyworm, *Spodoptera exigua*, it was shown that BPUs were mainly detoxified by hydrolytic cleavage and conjugation reactions involving mixed function oxidases (Van Laecke and Degheele 1991). Furthermore, it was demonstrated that a large part of diflubenzuron was rapidly excreted. Similar results were obtained in omnivorous leafroller, *Platynota stultana*, where DEF® (S,S,S-tributylphosphorotrithioate), an esterase inhibitor, synergized diflubenzuron suggesting that esterases are involved in the metabolic fate of BPUs (Granett and Hejazi 1983). Ishaaya and Klein (1990) suggested that multi-resistance factors caused by various groups of insecticides may confer tolerance to teflubenzuron in *Spodoptera littoralis* (Boisduval) as DEF synergized the toxicity of teflubenzuron in the resistant field strain which restored the insects susceptibility to this chemical. However, the more stable BPUs such as chlorfluazuron and teflubenzuron were synergized by esterase inhibitors to a much lesser extent than diflubenzuron in *S. littoralis* (El Saidy *et al.* 1989).

Perng *et al.* (1988) proposed that the increase in microsomal oxidation is the major mechanism of teflubenzuron resistance in DBM as the ovicidal effect of this compound was reduced when synergising with piperonyl butoxide (PBO). An increase in microsomal monooxygenase activities was also found in teflubenzuron resistant strains of DBM (Lin *et al.* 1989). The lack of significant cross-resistance to other BPUs indicates that different microsomal oxidases might be involved (Perng *et al.* 1988, Lin *et al.* 1989). Furthermore, it was shown that a higher carboxyamidase activity is involved in chlorfluazuron resistance in DBM as indicated by the metabolites which were obtained by carboxamide cleavage when studying the *in vivo* metabolism of ^{14}C-chlorfluazuron (Miyata *et al.* 1997).

In another research paper, it was suggested that GST-3 is involved in chlorfluazuron detoxification in DBM and it was demonstrated that the higher GST-3 expression in the resistant strain in comparison to the susceptible counterpart may result from enhanced gene transcription rather than gene amplification or point mutations in the structural gene (Sonoda and Tsumuki 2005).

In summary, multiple metabolic detoxification processes may play an essential role in BPU resistance as shown in *S. exigua* where a diverse range of enhanced detoxifying enzymes including GSTs, *O*-demethylase, microsomal oxidases and carboxyamidases may have contributed to a reduced susceptibility to a wide range of insecticides (Van Laecke *et al.* 1995).

In contrast to the many records of metabolic resistance, a single case of target-site mutation has been reported that confers BPU insensitivity in DBM. Douris, Steinbach *et al.* (2016) identified an amino acid substitution from isoleucine to methionine or phenylalanine at position 1042 in a highly conserved region in CHS1. This I1042M/F mutation was inherited in an autosomal recessive manner and resulted in high levels of resistance against triflumuron as well as cross-resistance to other BPUs tested. Furthermore, the mutation was present at relatively high frequencies in field populations of DBM sampled from cabbage fields in China and India with known BPU control failures and in a laboratory strain from Japan at a low frequency (Douris, Steinbach *et al.* 2016). The fact that the I1042F and I1042M mutation was found in several geographical regions indicates that it evolved independently.

1.6 Integrated Pest Management (IPM)

Chemical control measures have become more difficult due to the increasing issue of resistance which can deprive the utility of whole classes of insecticides when the resistance becomes severe. Additionally, the number of insecticides that are being commercialised are declining and it has become harder to introduce new insecticides with a novel MoA to the market. Therefore, it is important to delay the occurrence of resistance and thereby increasing the life span of an insecticide.

Integrated pest management (IPM), also known as integrated pest control (IPC), focuses on long-term pest prevention on an ecosystem-based strategy by combining different techniques that include biological control, the manipulation of habitats, the application of alternative pesticides, modification of cultural practices and the usage of resistant plant varieties. This is in contrast to traditional pest control which involves frequent pesticide application on a routine basis. In IPM systems pesticides are used rationally and carefully in conjunction with other technologies (Cooper and Dobson 2007) and uses monitoring programmes to improve the timing of pest suppression treatments. The application of chemical measures is conducted in a manner that reduces the risks to humans, beneficial as well as non-target organisms, and the environment.

During the past two decades, IPM programmes have reduced pest control costs and pesticide applications in fruit, vegetable and field crops (Popp *et al.* 2013). In order to improve pest control and thereby lessen yield losses, the integration of new scientific knowledge and modern technologies offers substantial opportunities as well as cost-effective and environmentally friendly solutions.

1.7 Objective of this Study

Sustainable resistance management strategies have become very important for modern agriculture as insecticide resistance against new chemical substances (such as diamides) is spreading vastly in lepidopteran pests. At the same time, resistant alleles that confer resistance against old classes of chemistries (BPUs) might still be present in a low frequency in a pest population. In contrast, the availability of new compounds with new MoAs is limited therefore, it is important to use our resources in an optimal way.

In order to successfully implement resistance management strategies, it is crucial to determine the extent/scope of the resistance at a geographical level, to identify and to understand the molecular mechanisms that underlie the resistance and to assess alternative chemical control measures as cross-resistance to other chemical classes with the same MoA needs to be avoided.

This thesis aims at different topics in a number of individual chapters in order to cover resistance mechanisms that lead to diamide and BPU resistance in lepidopteran pests.

Chapter 2

The objective of this study was to investigate and characterize the ryanodine receptor (RyR) mutation G4946E that confers high levels of diamide resistance in diamondback moth (DBM). Thus, the genetics of mutation G4946E was determined and functional evidence was provided by displaying the implication of the mutation on diamide binding to the insect RyR using radiolabelled binding assays. Additionally, a computational homology modelling approach was chosen to reveal the location of the mutation and another recently described mutations in the RyR transmembrane region specifying diamide binding region. Furthermore, genotyping of individual DBM populations worldwide was conducted in order to correlate field failures and the presence of the G4946E mutation.

Chapter 3

In this chapter the molecular mechanisms underlying diamide resistance in highly diamide resistant strains of *T. absoluta* were investigated. Synergism studies were performed assessing metabolic resistance. Reciprocal crosses were conducted to study the genetics of resistance. Gene sequencing of RyR and genotyping of the RyR transmembrane region was used to identify target-site mutations in *T. absoluta*. Following this approach, radioligand binding studies were

used to provide functional evidence that the mutations found in the RyR altered the affinity of the RyR to diamides.

Chapter 4

This chapter aimed to decipher the molecular mechanism underlying BPU resistance in a laboratory selected BPU resistant strain of DBM originating from the Philippines. Simultaneously the old enigma of MoA of selective chitin biosynthesis inhibitors in arthropods was unravelled. Classic genetic crossing experiments were conducted to provide insights into the genetics of resistance. Gene sequencing of chitin synthase 1 was performed in order to identify target-site mutations. Moreover, genome-editing of *Drosophila melanogaster* using CRISPR/Cas9 approach coupled with homology-directed repair (HDR) was conducted to provide functional evidence on the interactions and target-site of BPU.

Chapter 5

The aim of this chapter was the investigation of fitness costs, i.e. negative trade-offs, that were involved in insecticide resistance in different DBM strains which had shown resistance against diamides, BPUs and organophosphates. Life table studies at three different temperatures were performed in order to reveal negative trade-offs in a more extreme/stressful environment.

1.8 References

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Chapter 2

Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, *Plutella xylostella*

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Abstract

Anthranilic diamides and flubendiamide belong to a new chemical class of insecticides acting as conformation sensitive activators of the insect ryanodine receptor (RyR). These compounds control a diverse range of different herbivorous insects including diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), a notorious global pest on cruciferous crops, which recently developed resistance due to target-site mutations located in the trans-membrane domain of the *Plutella* RyR. In the present study we further investigated the genetics and functional implications of a RyR G4946E target-site mutation we recently identified in a Philippine diamondback moth strain (Sudlon). Strain Sudlon is homozygous for the G4946E mutation and has been maintained under laboratory conditions without selection pressure for almost four years, and still exhibit stable resistance ratios of >2000-fold to all commercial diamides. Its F1 progeny resulting from reciprocal crosses with a susceptible strain (BCS-S) revealed no maternal effects and a diamide susceptible phenotype, suggesting an autosomally almost recessive mode of inheritance. Subsequent back-crosses indicate a near monogenic nature of the diamide resistance in strain Sudlon. Radioligand binding studies with *Plutella* thoracic microsomal membrane preparations provided direct evidence for the dramatic functional implications of the RyR G4946E mutation on both diamide specific binding and its

concentration dependent modulation of [³H]ryanodine binding. Computational modelling based on a cryo-EM structure of rabbit RyR1 suggests that *Plutella* G4946E is located in transmembrane helix S4 close to S4–S5 linker domain supposed to be involved in the modulation of the voltage sensor, and another recently described mutation, I4790M in helix S2 approx. 13 Å opposite of G4946E. Genotyping by pyrosequencing revealed the presence of the RyR G4946E mutation in larvae collected in 2013/14 in regions of ten different countries where diamide insecticides largely failed to control diamondback moth populations. Thus, our study highlights the global importance of the G4946E RyR target-site mutation, which as a mechanism on its own, confers high-level resistance to diamide insecticides in diamondback moth.

2.1 Introduction

Diamide insecticides represent the most recent class of chemistry (reviewed in Jeanguenat 2013) introduced to the market to control a broad range of herbivorous pest insects, particularly of the order Lepidoptera (Nauen 2006, Hirooka *et al.* 2007, Lahm *et al.* 2007). Three compounds, i.e. the phthalic diamide flubendiamide and the anthranilic diamides chlorantraniliprole and cyantraniliprole, were commercialized with a global turn-over of >1.4 bn USD representing approx. 8 % of the insecticide market in 2013 (Sparks and Nauen 2015).

Typical symptoms of poisoning after application of diamide insecticides to lepidopteran larvae include rapid feeding cessation, followed by contraction paralysis and death (Tohnishi *et al.* 2005). Electrophysiological, biochemical and radioligand binding studies on the mode of action of these compounds revealed that they act as conformation sensitive activators of insect ryanodine receptors (RyR) by binding to a site different from ryanodine (and caffeine), present in the endo/sarcoplasmic reticulum of nerve and muscle tissue (Ebbinghaus-Kintscher *et al.* 2006, Cordova *et al.* 2006, Lümmen *et al.* 2007, Sattelle *et al.* 2008). RyRs are large homotetrameric, ryanodine-sensitive calcium release channels endogenously activated by Ca^{2+}-influx, mediated by voltage-gated calcium channels upon depolarization of the cell membrane (Lümmen 2013). Calcium is a universal intracellular messenger and the activation of RyRs triggers massive Ca^{2+}-efflux from intracellular stores into the cytosol resulting in physiological responses such as muscle contraction. Unlike mammals which possess three RyR isoforms (RyR1-3) localized in different tissues (Rossi and Sorrentino 2002), insect RyRs are encoded by a single gene with an ORF of >15,000 bp encoding a protomer of more than 500 kDa as first described for *Drosophila melanogaster* (Takeshima *et al.* 1994). Amino acid based sequence identity between insect RyRs and mammalian RyR isoforms is around 45 %, as recently shown for a number of insects including those targeted by diamides (Sattelle *et al.* 2008, Kato *et al.* 2009, Wang *et al.* 2012). RyRs of both vertebrates and insects were shown to be composed of a large N-terminal cytosolic domain and six helical transmembrane spanning domains at the C-terminal end of the receptor containing the ion-conducting pore (Xu *et al.* 2000, Lümmen 2013).

A recently published structure of a mammalian RyR1 determined by single-particle electron cryomicroscopy provided interesting insights regarding its structural features as it resolves in total 70 % of 2.2 MDa molecular mass homotetrameric channel protein (Yan *et al.* 2015).

Both flubendiamide and anthranilic diamides show nanomolar specificity for insect RyR´s and do barely affect isoforms of their mammalian counterparts, thus explaining their excellent safety profile (Ebbinghaus-Kintscher *et al.* 2006, Sattelle *et al.* 2008). Highly specific RyR binding sites for diamide insecticides allosterically stimulating [³H]ryanodine binding were only found in insect preparations (Qi and Casida 2013). Diamide structure-activity relationships in *Heliothis virescens* (tobacco budworm) thoracic muscle preparations recently indicated that both [³H]chlorantraniliprole and [³H]flubendiamide address a common binding site (Qi *et al.* 2014). When employing a photoreactive derivative of flubendiamide against a series of *Bombyx mori* RyR deletion mutants recombinantly expressed in HEK293 cells, Kato *et al.* (2009) concluded that the diamide binding site is likely to be located in the C-terminal transmembrane spanning domain. Further evidence for a critical role of this region for anthranilic and phthalic diamide binding was provided by a study replacing a 46 amino acid segment in the *Drosophila* RyR C-terminal domain by that of a nematode RyR which resulted in insensitivity to diamides (Tao *et al.* 2013).

Flubendiamide and chlorantraniliprole received their first registrations for field use in the Philippines in 2006 and 2007, respectively. Meanwhile diamide insecticides are globally used both solo and in mixtures by millions of farmers for foliar, drench and seed treatment applications in a broad range of agricultural and horticultural cropping systems, thus facilitating the evolution of insect resistance due to increasing selection pressure, particularly on lepidopteran pests (Teixeira and Andaloro 2013). Recently conducted baseline studies and resistance monitoring campaigns confirmed the regional development of diamide resistance in a few pests, but resistance ratios vary between species. Very high resistance ratios leading to diamide field failure were published for diamondback moth, *Plutella xylostella* (Troczka *et al.* 2012, Wang and Wu 2012, Ribeiro *et al.* 2014), tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014) and tomato leafminer, *Tuta absoluta* (Roditakis *et al.* 2015). Whereas low to moderate resistance ratios were reported for rice stem borer, *Chilo suppressalis* (Gao *et al.* 2013, He *et al.* 2014), beet armyworm, *Spodoptera exigua* (Che *et al.* 2013), rice leaffolder, *Cnaphalocrocis medinalis* (Zhang *et al.* 2014) and the obliquebanded leafroller, *Choristoneura rosaceana* (Sial *et al.* 2011).

The currently best documented case of diamide resistance involves diamondback moth, a global lepidopteran pest of cruciferous vegetables. It is known as a notorious candidate for rapid resistance development to almost all chemical classes of insecticide applied for its control, particularly in (sub-) tropical areas with intensive use of crop protection products (Talekar and Shelton 1993, Teixeira and Andaloro 2013). High levels of diamide resistance in *P. xylostella*

were first reported in Asian strains sampled in the Philippines, Thailand and China (Troczka *et al.* 2012, Wang and Wu 2012), but very recently also in populations collected in Brazil (Ribeiro *et al.* 2014). Diamide resistance was shown to be unstable, partly metabolically driven, autosomal and incompletely recessive in a Chinese population (Wang *et al.* 2013), whereas unstable resistance and fitness costs due to reduced fecundity was described for a Brazilian population (Ribeiro *et al.* 2014). Early studies on the mechanisms of diamide resistance conducted in two diamondback moth strains collected in the Philippines and Thailand revealed an amino acid substitution G4946E in the C-terminus of the *Plutella* RyR (Troczka *et al.* 2012), when compared to a RyR cloned from an insecticide susceptible strain (Wang *et al.* 2012). Recently a few more mutations were described in a diamide resistant strain of diamondback moth from China, two of them, I4790M and Q4594L were shown to be present in the C-terminal domain of the RyR supposed to harbor the diamide binding site (Guo *et al.* 2014a). Attempts to demonstrate any functional implications of the G4946E mutation for diamide binding by *in vitro* radioligand binding studies were not yet published. However, recent *in vitro* studies using a diamide-like fluorescent tracer (Wang *et al.* 2014) in fluorescence polarization binding assays only revealed slight affinity differences of less than 2.5-fold (based on K_D-values) between membranes isolated from susceptible and resistant diamondback moth populations showing resistance ratios of greater than 2000-fold (Guo *et al.* 2014a, Guo *et al.* 2014b). Considering the minor shift in K_D-value the study largely failed to provide functional evidence that the G4946E mutation confers a significant level of target-site resistance as one would deduce from bioassay data.

The present work aims to provide a deeper understanding of diamide insecticide resistance based on the recently described G4946E RyR mutation in a Philippine strain of diamondback moth, by providing data on the genetics of resistance, its global presence and finally its functional implications for diamide binding by radioligand binding studies. Furthermore we mapped the known mutations to a *Plutella* RyR homology model based on newly published structural information of rabbit RyR1 (Yan *et al.* 2015).

2.2 Material and methods

2.2.1 Chemicals

Insecticides (flubendiamide, chlorantraniliprole, cyantraniliprole) were of technical grade (purity >97 %) and provided in-house (Bayer CropScience, Monheim). [³H]Ryanodine (spec. activity 2 TBq mmol⁻¹) and the des-methylated flubendiamide derivative [³H]PAD1 [3-iodo-N-(2-methanesulfonyl-1-methyl-ethyl)-N´-[2-methyl-4-(1,2,2,2-tetrafluoro-1-trifluoromethyl-ethyl)-phenyl]-phthalamide] (spec. activity 777 GBq mmol⁻¹) were purchased from PerkinElmer

and Amersham Biosciences, respectively. All other chemicals and solvents were of analytical grade and purchased from Sigma Aldrich (Steinheim, Germany).

2.2.2 Insects

The susceptible reference strain (BCS-S) of *Plutella xylostella* L. (Lepidoptera: Plutellidae) was reared on cabbage plants (*Brassica oleracea*) in plastic cages at 23 ± 1 °C with a relative humidity of 60 ± 10 % under blue light. It has been maintained under laboratory conditions for more than 20 years without exposure to insecticides. The diamide resistant strain (Sudlon) was collected in a cabbage field located in Sudlon, Cebu Island, in the Philippines in 2011 and also maintained on cabbage, but kept separately at 23 ± 1 °C, relative humidity 50 ± 10 % with an ambient photoperiod (exception permit SG 63/10-DE-NW for rearing was provided by the chamber of agriculture, NRW, Germany). Strain Sudlon was recently described to be homozygous for a point mutation in the diamondback moth RyR gene, resulting in a G4946E amino acid substitution in the transmembrane region of the RyR channel protein (Troczka *et al.* 2012). The organophosphate and pyrethroid resistant strain (Japan) was collected in Mizobe, Japan in 2010 and was reared on cabbage in another rearing room under the same conditions as mentioned above. Both strains, Sudlon and Japan were not kept under insecticide selection pressure. Adults of *P. xylostella* were fed with 10 % (w/v) sucrose solution.

Furthermore, diamondback moth larvae were field-collected in 11 different countries, preserved in alcohol and shipped to the laboratory of the corresponding author. All samples were derived from cabbage fields where diamide insecticides showed either reduced efficacy (Australia, Indonesia, India (Karnataka) and Korea (Bongnam-ri)) or largely failed to control diamondback moth larvae at manufacturer recommended rates (India (Tamil Nadu), Japan, Korea (Sangan-ri), Philippines, Taiwan, Thailand, USA and Vietnam). We also analysed alcohol preserved samples collected from different cabbage fields in four different Chinese provinces, i.e. Fujian, Guangdong, Guangxi and Shandong. At the time of collection (2013) diamide field failure was observed in all provinces, except Shandong (Quansheng Hu, personal communication).

2.2.3 Bioassay method

Leaf dip bioassays were conducted after IRAC Method No. 7 (www.irac-online.org). Technical grade insecticides were dissolved in acetone and diluted in aqueous 0.1 % triton X-100 (v/v). For each insecticide at least six insecticide dilutions were used to estimate LC_{50}-values. After dipping, leaves were dried on filter paper and placed in petri dishes. The leaves were infested with 10 third instar larvae and each concentration was replicated thrice. After 72 h at 22 ± 1 °C affected larvae were assessed, i.e. those showing symptoms of poisoning and death. Larvae feeding on aqueous triton X-100-treated leaves served as control and showed a mortality of less

than 10 % in all bioassays. LC_{50}-values and 95 % confidence limits were calculated using Graph Pad Prism 5.03 (GraphPad Software, Inc., USA).

2.2.4 Crossing experiments and genetics of resistance

Strain Sudlon was recently shown to be homozygous (RR) for a G4946E mutation in the transmembrane domain of the diamondback moth RyR and highly resistant to diamide insecticides (Troczka *et al.* 2012). Pupae of BCS-S and Sudlon strain were collected and kept in petri dishes individually until adult emergence. After sex determination, 50 virgin females of BCS-S strain were crossed with 50 males of Sudlon strain and *vice versa*. Since there was no diamide toxicity difference observed between the resulting F1 obtained from reciprocal crosses, F1 generation was pooled for further studies. Subsequently the F1 generation was back-crossed with parental strains to check for monogenic resistance. Expected mortality for a monogenic model was calculated using the following formula; expected % mortality F1 at [c] = 0.5 x (% mortality of F1 at [c] + % mortality of strain Sudlon at [c]), where [c] refers to the respective concentration of the insecticide. Third instar larvae of each genetic cross were tested in leaf dip bioassays to obtain LC_{50}-values for both flubendiamide and chlorantraniliprole. The degree of dominance (D) was calculated using Stone's formula (Stone 1968) $D = (2X_2 - X_1 - X_3)/(X_1 - X_3)$, where X_1, X_2 and X_3 are logarithms of LC_{50} values for RR (Sudlon), RS (F1 generation) and SS (BCS-S) strains. Larvae of the different strains were kept in RNA*later*® (Ambion) solution and screened for the presence of the G4946E mutation by pyrosequencing.

2.2.5 Pyrosequencing method

The recently published pyrosequencing method (Troczka *et al.* 2012) was optimized for using genomic DNA rather than RNA in order to reduce the costs for molecular diagnostics. Briefly: Genomic DNA from individual adults or larvae of *P. xylostella* was extracted using DNAdvance Tissue Kit (Agencourt) according to the supplier's recommended protocol. A short gene fragment of 79 bp was amplified by PCR from 50 ng aliquots of gDNA using the new primer pair 494-for (5´-GCCGCTCATCTGTTGGACGT-3´) and 536-rev_btn (5´-[btn]TCCCRTTATGYRTGACRGAC-3´), designed with Assay Design Software (PSQ-Biotage AB, now Qiagen). The primer pair is based on a ClustalW aligned consensus sequence using several diamondback moth RyR genes found in GenBank (JQ769303.1, JN801028.1, JF926693.1, JF926694.1) as well as internally sequenced strains from Japan, Philippines, India, Taiwan and Indonesia (data not shown). The pyrosequencing protocol comprised 40 PCR cycles with 0.5 μM forward and biotinylated reverse primer in 50 μL reaction mixtures containing $1 \times$ Taq enzyme reaction mix (RedTaq Jumpstart Master Mix, Sigma Aldrich) and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C

for 45 s, and a final elongation step at 72 °C for 5 min. The pyrosequencing reaction (incl. sequence-primer for genotyping) was carried out as recently described (Troczka *et al.* 2012).

2.2.6 Preparation of endoplasmic/sarcoplasmic reticulum (ER/SR) membranes

Diamondback moth adults were quickly frozen in liquid nitrogen and shaken vigorously to break off heads and abdomen. Thoraces were collected on analytical sieves (1.4 mm, 1.0 mm, 0.5 mm diameter). One g thoraces per 20 mL buffer (25 mM K-PIPES, pH 7.4, 0.3 M sucrose, 0.2 M KCl, 0.1 mM EDTA, 1 mM DTT, protease inhibitor cocktail Halt™, Thermo/Pierce) were homogenized using an UltraTurrax. After centrifugation for 10 min at 1,000g, the supernatant was filtered through five layers of cheesecloth. The pellet was re-homogenized and centrifuged as described above. The combined supernatants were centrifuged for 20 min at 8,000g and the pellet was discarded. The supernatant was again filtered through five layers of cheesecloth and centrifuged for 60 min at 100,000g to collect ER/SR membranes. The sediment was resuspended in a small volume of 20 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 1.0 mM DTT, 0.8 % (w/v) BSA using a Potter homogenizer. The protein concentration (Bradford method) was adjusted to 0.5 mg mL^{-1}.

2.2.7 Radioligand binding studies

Equilibrium binding assays were performed in 10 mM HEPES, pH 7.4, 0.8 mM CaCl$_2$, 1.5 M KCl, 10 mM ATP, in 96-well plates. The concentration of ER/SR membrane protein was 50 μg in a total assay volume of 250 μL. Enhancement of ryanodine binding was measured using 4.0 nM [^{3}H]ryanodine and variable concentrations of the diamide compounds, chlorantraniliprole and flubendiamide in binding buffer including 0.02 % Pluronic® to increase the solubility of the test compounds. Following 2 h incubation at 25 °C, assay mixtures were filtered through UniFilter-96 GF/B plates (PerkinElmer) pre-wetted with 0.1 % polyethyleneimine using a Brandel cell harvester. Filters were washed twice with 1 mL ice-cold 10 mM HEPES, pH7.4, 1.5 M KCl. Radioactivity retained on the filters was measured for 2 min by scintillation counting (TopCount, PerkinElmer). Non-specific binding was determined with 10 μM unlabeled ryanodine.

Equilibrium saturation binding of [^{3}H]PAD1 was measured essentially as described before, but the radioligand concentrations were varied between 0.1 and 60 nM. Non-specific binding was measured with 10 μM unlabeled PAD1. Equilibrium binding parameters were calculated using the SigmaPlot 11.0 software (SPSS).

2.2.8 Computational modelling

Taking into account the publication of Yan *et al.* (2015) who described the conformational model of the European rabbit RyR1 in complex with FKBP12 at 3.8 Å resolution determined by single-particle cryo-electron microscopy (cryo-EM), a multiple amino acid alignment of the RyR wildtype sequence of *P. xylostella* (UniProt G8EME3; Guo *et al.* 2012) and the rabbit structure (PDB 3J8H) was used to map the known mutation sites linked to diamide resistance (Troczka *et al.* 2012, Guo *et al.* 2014). As PDB 3J8H does not cover the complete sequence of the rabbit RyR1 (due to structural disorder), another but complete sequence of *Oryctolagus cuniculus* RyR1 (UniProt P11716) was added to the pairwise alignment to map the gaps in the structure determined by cryo-EM. Computational modelling illustrations including the mutation sites were generated using the SYBYL software package (SYBYL X2.1.1; Certara, L.P.: St. Louis MO, 2014).

2.3 Results

2.3.1 Bioassays

Log-dose probit-mortality data obtained from leaf dip bioassays with third instar diamondback moth larvae of strains BCS-S, Japan and Sudlon are given in Table 2.1. Strain Sudlon shows resistance ratios of >10,000-fold and approx. 2600-fold against flubendiamide or chlorantraniliprole and cyantraniliprole, respectively. Cyantraniliprole shows a somewhat higher intrinsic efficacy in our bioassay set up than flubendiamide or chlorantraniliprole against all strains tested. LC_{50}-values against the Japan strain are similar to the ones of the reference BCS-S strain, confirming its susceptibility towards diamide insecticides.

Table 2.1. Log-dose probit-mortality for commercial diamide insecticides tested against 3rd instar larvae of different strains of diamondback moth in leaf-dip bioassays (72h).

Compound Strain	n	LC_{50} [mg L^{-1}]	CL95%[a]	Slope	RR[b]
Flubendiamide					
BCS-S	360	0.029	0.026-0.033	1.9	
Japan	360	0.015	0.0095-0.025	1.1	
Sudlon	120	>1000			>10,000
Chlorantraniliprole					
BCS-S	480	0.020	0.013-0.031	0.75	
Japan	480	0.045	0.032-0.064	0.75	
Sudlon	180	>1000			>10,000
Cyantraniliprole					
BCS-S	420	0.0068	0.0039-0.012	1.9	
Japan	420	0.0045	0.0039-0.0052	2.2	
Sudlon	240	18	5.1-66	1.0	2647

[a] 95% confidence limits

[b] Resistance ratio (LC_{50} of Sudlon strain divided by LC_{50} of BCS-S strain)

3.3.2 Genetics of diamide resistance

Bioassay data for the parental strains BCS-S and Sudlon as well as their reciprocal crosses (combined F1) and the backcross strains (F1 x BCS-S and F1 x Sudlon) for both flubendiamide and chlorantraniliprole are shown in Table 2.2. No difference in LC_{50}-values was observed between the resulting F1 progeny of reciprocal crosses (data not shown), therefore log-dose probit mortality data were combined and expressed as a composite value (BCS-S x Sudlon (F1), Table 2.2). The fact we observed no maternal effects suggests that diamide resistance in strain Sudlon is autosomally inherited. The calculated composite LC_{50}-values of the F1 progeny for flubendiamide and chlorantraniliprole were 0.078 mg L^{-1} and 0.064 mg L^{-1}, respectively and clearly suggest a diamide susceptible heterozygous phenotype. The calculated degree of dominance (D) of -0.85 and -0.78 for flubendiamide and chlorantraniliprole, respectively, supports the result and indicates an almost completely recessive mode of inheritance. Furthermore, larvae of F1 backcrosses were tested with flubendiamide, and the resulting dose response of the F1 x Sudlon progeny matches the expected mortality for near monogenic resistance (showing a plateau at 50% mortality; Fig. 2.1). Genotyping by pyrosequencing of the F1 x Sudlon backcross revealed that 42 % and 58 % of the tested larvae (n = 50) were homozygous and heterozygous for the G4946E mutation in the *Plutella* RyR, respectively. Whereas the F1 x BCS-S backcross progeny (n = 40) include 80 % susceptible homozygotes and 20 % heterozygotes, exhibiting a similar susceptibility to flubendiamide as the BCS-S strain (Table 2.2, Fig. 2.1).

Table 2.2 Log-dose probit-mortality data for flubendiamide and chlorantraniliprole tested against crosses and backcrosses of 3rd instar larvae of diamondback moth strains BCS-S and Sudlon.

Compound	Strain	n	LC_{50} [mg L^{-1}]	CL95%[a]	Slope	D[b]

Flubendiamide	BCS-S	360	0.028	0.026-0.034	1.9	
	Sudlon	120	>1000			
	F1 (combined)	540	0.078	0.053-0.10	1.2	-0.81
	F1 x BCS-S	540	0.028	0.023-0.033	1.5	
	F1 x Sudlon	540	Ambiguous			
Chlorantraniliprole	BCS-S	270	0.020	0.013-0.031	0.75	
	Sudlon	120	>1000			
	F1 (combined)	240	0.064	0.055-0.075	1.3	-0.78

[a] 95% confidence limits

[b] Degree of dominance
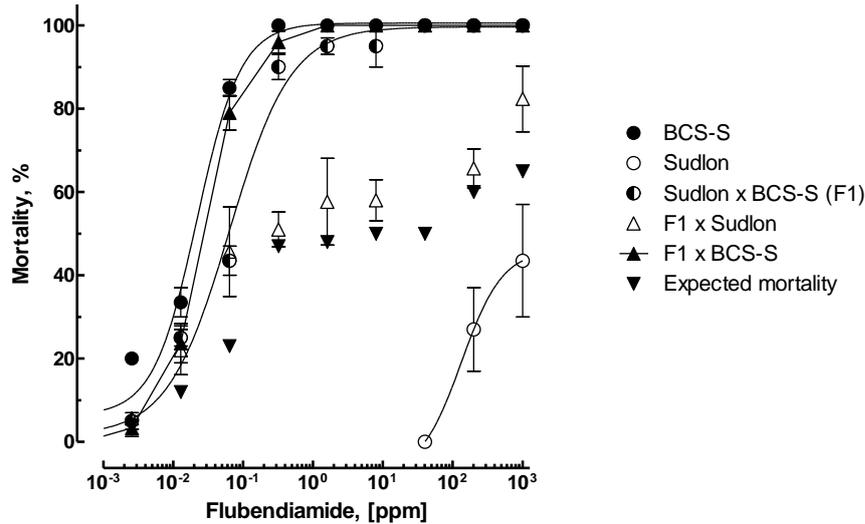
Figure 2.1. Log-dose response curves for flubendiamide tested against 3rd instar diamondback moth larvae of strains BCS-S, Sudlon, combined reciprocal crosses BCS-S x Sudlon (F1 hybrids), F1 x BCS-S backcross, F1 x Sudlon backcross and its calculated theoretical backcross based on monogenic resistance. Data obtained for F1 hybrids resulting from reciprocal crosses were pooled because no maternal effects were seen.

3.3.3 Genotyping by pyrosequencing

We analysed alcohol preserved diamondback moth larvae (n = 400) obtained from 11 different countries and collected between 2012 and 2014, for the presence of the G4946E mutation by pyrosequencing of a partial 72bp sequence of amplified genomic DNA. The pyrosequencing assay based on genomic DNA worked well for 95 % of all samples analysed. Genotypes homozygous for the G4946E mutation were found in 9 out of 11 eleven countries (Table 2.3).

Table 2.3. Genotyping by pyrosequencing for a RyR target-site mutation (G4946E) in diamondback moth larvae sampled in cabbage fields in 2014 with diamide insecticide control failures in different countries. For each sampling site 12-24 larvae were analysed.

Country	Region	Sampling sites	G4946E genotype (%)		
		(n)	RR	SR	SS
Australia	Queensland	(4)	0	0	100
India	Karnataka	(2)	0	33	67
	Tamil Nadu	(1)	50	25	25
Indonesia	Central Java	(1)	0	67	33

	Bali	(2)	0	50	50
Japan	Miyazaki	(1)	88	12	0
	Chiba	(1)	50	50	0
Korea	Bongnam-ri	(1)	0	12	88
	Sangan-ri	(1)	70	30	0
Philippines	Calauan	(1)	85	15	0
Thailand	Bang Bua Thong	(1)	90	10	0
USA	Mississippi	(1)	100	0	0
Vietnam	South East	(1)	100	0	0
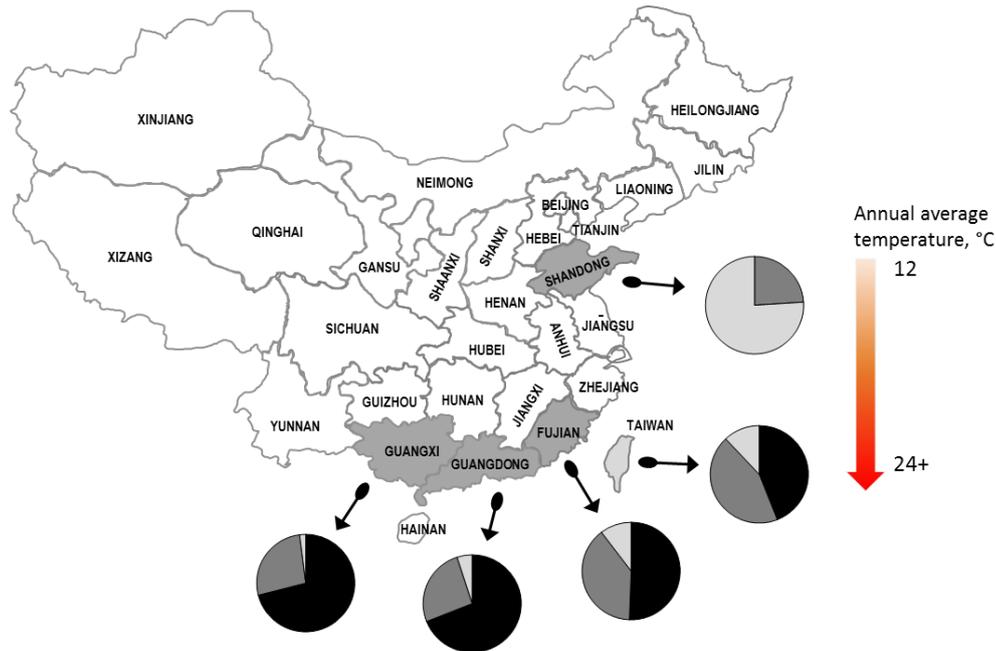
Figure 2.2. Genotyping for RyR target-site mutation (G4946E) in diamondback moth larvae sampled in diamide insecticide treated cabbage fields in different provinces in China and Taiwan in 2013. Pie charts display the proportion of genotypes assigned to RR resistant homozygotes (black), SR heterozygotes (dark grey) and SS susceptible homozygotes (light grey). Data obtained from 12-24 larvae analysed per collection site (i.e. fields) and region were combined, i.e. Guangxi (3 sites), Fujian (3), Guangdong (5) and Shandong (3). In Taiwan 8 different sites were sampled and combined. The arrow displays the annual average temperature gradient between provinces Shandong in the north east and Guangxi, Guangdong and Fujian in the south of China, as well as Taiwan.

All these samples were collected in regions where diamides failed to control diamondback moth populations at recommended rates, whereas populations collected in regions where diamides still provide a certain level of control include mostly heterozygous and homozygous susceptible genotypes (e.g. Australia and Indonesia). We also analysed several populations collected in Taiwan (n = 8) and different Chinese provinces such as Guangxi (3), Guangdong (5), Fujian (3) and Shandong (3). Combined data for all populations analysed from Taiwan, Guangxi, Guangdong and Fujian revealed only a small proportion ($\leq$15 %) of genotypes homozygous for wildtype G4946, thus confirming high levels of diamide resistance (Fig. 2.2). In contrast approx. 75 % and 25 % of diamondback moth larvae analysed from Shandong province still

represent wildtype G4946 and heterozygotes, respectively. Thus corresponding to the observation that diamides in most places in Shandong province still worked well in 2014 (Quansheng Hu, personal communication).

3.3.4 Radioligand binding studies

Equilibrium saturation kinetics using the des-methylated flubendiamide analogue [³H]PAD1 in the presence of 800 μM calcium, revealed highly specific binding to thoracic muscle membrane preparations of the diamide susceptible diamondback moth strain BCS-S. Non-specific binding of [³H]PAD1 to *Plutella* BCS-S thoracic membrane preparations is less than 10 % (data not shown). The apparent dissociation constant K_D shows a value of 2.7 ± 0.23 nM (Hill coefficient 1.0) and the receptor density expressed in B_{max} was determined at 8.3 ± 0.19 pmol mg^{-1} (Fig. 2.3). However membrane preparations of the diamide resistant strain Sudlon did not show specific saturable [³H]PAD1 binding using the same assay protocol.
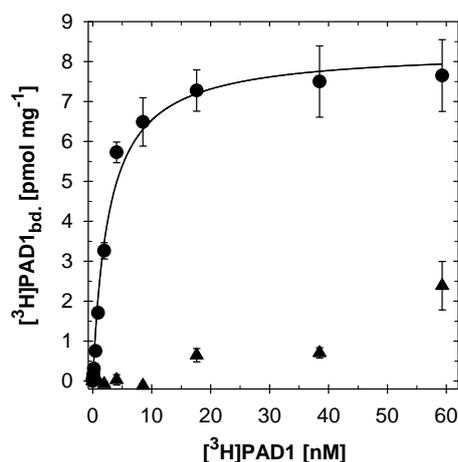
Figure 2.3. Specific (saturation) binding of the flubendiamide derivative [³H]PAD1 to thoracic microsomal membranes of susceptible (BCS-S) (●) and resistant (Sudlon) (▲) diamondback moth adults. Data are mean values ± SD (n = 3).
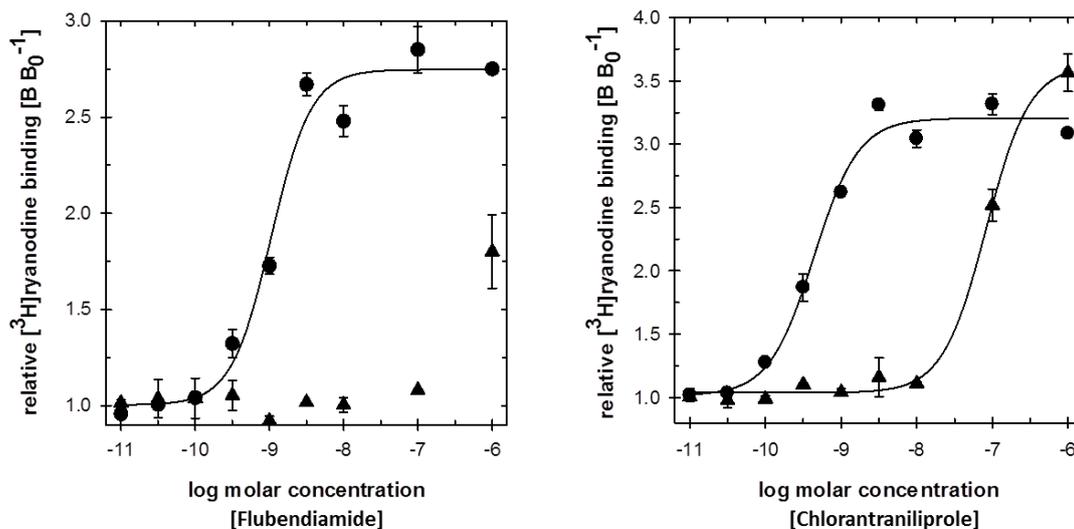
Figure 2.4. Relative increase of [³H]-ryanodine binding to thoracic microsomal membranes of susceptible (BCS-S) (●) and resistant (Sudlon) (▲) diamondback moth adults as a function of diamide insecticide concentration reveal functional implications of the G4946E mutation for diamide binding. Data are mean values ± SD (n = 3).

Under the assay conditions used in the present study both flubendiamide and chlorantraniliprole are known to act as positive allosteric activators of [³H]ryanodine binding to lepidopteran RyRs (Ebbinghaus-Kintscher *et al.* 2006). Radioligand binding studies using thoracic microsomal membrane preparations of strain BCS-S and Sudlon revealed a relative increase of [³H]ryanodine binding as a function of flubendiamide concentration at an EC_{50} of 1.1 ± 0.087 nM and > 500 nM (estimate, no curve fitting possible), respectively (Fig. 2.4). The same experimental set-up using chlorantraniliprole revealed EC_{50} values for the relative increase of [³H]ryanodine binding of 0.50 ± 0.089 nM and 79.4 ± 9.22 nM using membrane preparations of strain BCS-S and Sudlon, respectively. Thus demonstrating in strain Sudlon RyR target-site resistance ratios of >450 and 159 fold for flubendiamide and chlorantraniliprole, respectively.

3.3.5 Ryanodine receptor modelling

A recently published conformational model of the rabbit RyR1 obtained by single-particle cryo-electron microscopy (Yan *et al.* 2015) was used to map the recently described *Plutella* RyR G4946E and I4790M mutation sites linked to diamide insecticide resistance. The C-terminal domain revealed 58 % amino acid similarity between rabbit RyR1 (UniProt P11716) and *Plutella* RyR (UniProt G8EME3). Thus it was a straightforward approach to use a multiple sequence alignment together with the rabbit RyR1 parent structure without building an explicit RyR model for the *Plutella* mutants. Two of the known diamondback moth RyR mutations (G4946E, I4790M with a cysteine in rabbit RyR1) could be unambiguously located in the parent structure (Fig. 2.5). However, two additional mutations recently described by Guo *et al.* (2014)

could not be precisely mapped. *Plutella* RyR E1338D was mapped into an insert region not present in *O. cuniculus*, and Q4594L was missing in the corresponding PDB structure 3J8H due to disorder. According to the RyR structural model the mutations G4946E and I4790M are located in close proximity to each other in the voltage sensor domain (see modelled RyR protomer in Fig. 2.5): G4946E is located at the interface between the S4–S5 linker helix and the S4 helix, while I4790M is located directly opposite in the upper region of the S2 helix (Fig. 2.5). The distance between the respective Cα atom positions of the mutated amino acids is roughly 13 Å.
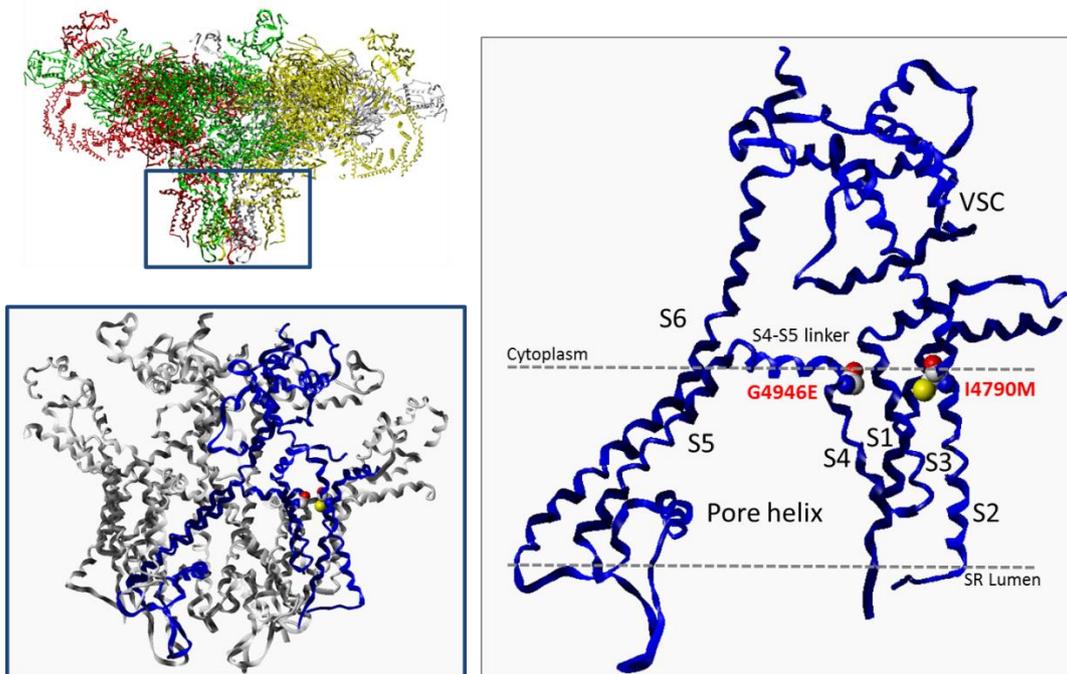
Figure 2.5. Positions of two recently described mutations in the RyR protomer (right) conferring diamide insecticide resistance in diamondback moth (Troczka *et al.* 2012, Guo *et al.* 2014). Based on rabbit RyR1 protomer modelling the mutations G4946E and I4790M are located in trans-membrane domains S4 and S2, i.e. close to the cytoplasmic voltage sensor sub-domain. The overall structure (top left) and the tetrameric trans-membrane domain (bottom left) is based on the recently published rabbit RyR1 structure (PDB code 3J8H) determined by single-particle cryomicroscopy (Yan *et al.* 2015).
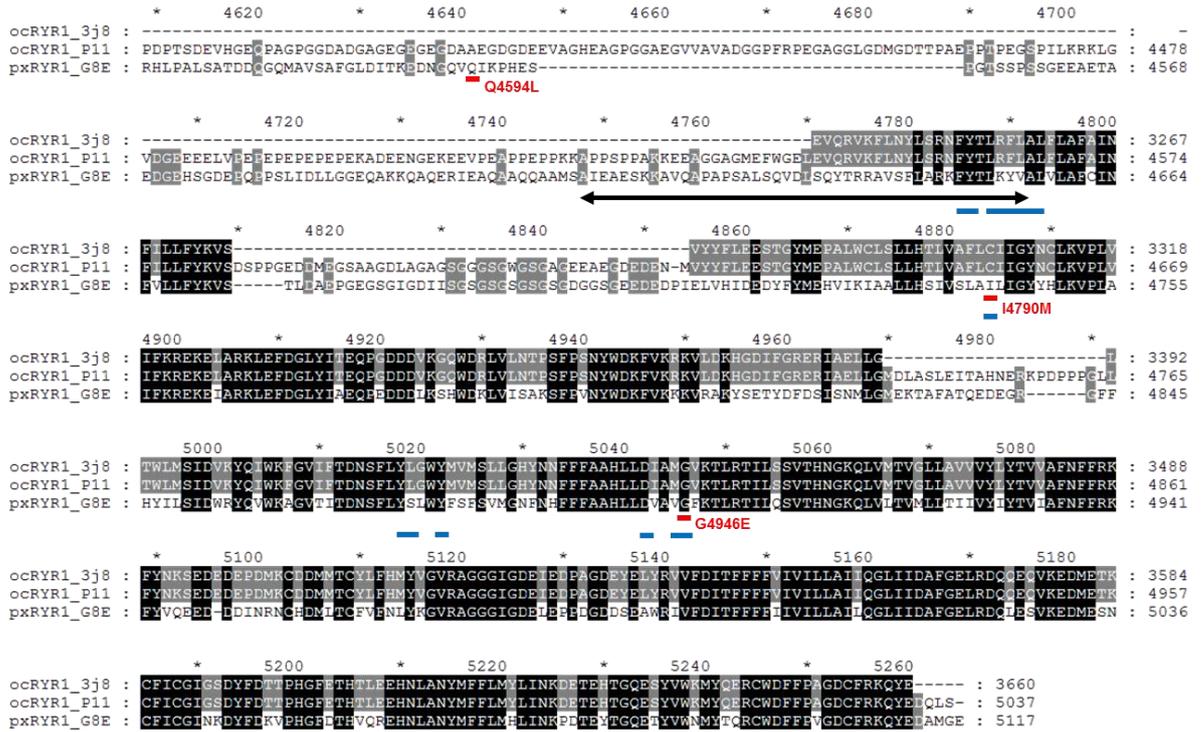
Figure 2.6. Amino acid alignment of partial RyR sequences of European rabbit RyR1 (PDB 3J8H and UniProt P11716) and diamondback moth RyR (UniProt G8EME3) covering the C-terminal trans-membrane domain. Conserved amino acid residues across species are highlighted in black. Mutation sites Q4594M, I4790M and G4946E recently described in the diamondback moth RyR and linked to diamide insecticide resistance are indicated by a red bar. Those amino acids marked by a blue bar are within a distance of approx. 7 Å from a midpoint center between Cα positions of I4790 and G4946 analogous residues in 3J8H (refer to Fig. 2.5), possibly describing the diamide binding site in insect RyRs. The black bar covers a critical 45 amino acid region of the *Drosophila* RyR recently described to be involved in diamide insecticide binding (Tao *et al.* 2013).

2.4 Discussion

It is foreseeable that the current global market share of 8 % for diamide insecticides will further increase as new anthranilic diamides such as cyclaniliprole and tetraniliprole are currently under development (Sparks and Nauen 2015). Diamide insecticides target insect RyRs and bind with nanomolar affinity to a site different from ryanodine (Cordova *et al.* 2006, Lümmen *et al.* 2007, Qi and Casida 2013), but allosterically coupled and located in the C-terminal membrane-spanning domain (Kato *et al.* 2009, Isaacs *et al.* 2012, Lümmen, 2013). However, the intense use of diamide insecticides, particularly against diamondback moth, which is known as one of the most devastating pests in cruciferous vegetables, led to resistance development seriously compromising their field efficacy, particularly in Asia (Teixeira and Andaloro 2013).

We recently linked a RyR target-site mutation present in a Philippine strain of diamondback moth to diamide insecticide cross-resistance (Troczka *et al.* 2012). In the same study we were

able to show that another non-synonymous mutation in a population from Thailand led to the very same amino acid substitution in a highly conserved region of the C-terminal trans-membrane spanning domain of the RyR protein, supposed to harbor the binding site of diamides (Kato *et al.* 2009). The SNP´s we found in both strains result in a G4946E substitution, and the fact it evolves independently in two different geographies supported our view it is directly involved in diamide cross-resistance. Meanwhile the G4946E mutation was also described to be present in Chinese populations of diamondback moth showing different levels of resistance to diamide insecticides, which is most likely driven by the proportion of G4946E RR homozygotes present in a population (Gong *et al.* 2014, Guo *et al.* 2014a and b, Yan *et al.* 2014). Whereas resistance to diamides was described to be unstable without selection pressure in populations from China (Wang *et al.* 2013) and Brazil (Ribeiro *et al.* 2014), the Philippine Sudlon strain investigated here, still show high levels of resistance even after maintaining it for almost four years under laboratory conditions. Bioassays conducted revealed resistance ratios of greater than 2000-fold against all diamides tested. Radioligand binding studies using a tritiated flubendiamide derivative [³H]PAD1 confirmed its high-affinity binding to diamondback moth thoracic muscle membrane preparations in the susceptible strain BCS-S. The apparent K_D-value of 2.7nM for [³H]PAD1 reported here for susceptible *Plutella* is in the same range as recently reported for saturation binding of [³H]flubendiamide in *H. virescens* microsomal membranes (K_D 3.4 nM) (Lümmen *et al.* 2007). However using [³H]PAD1 in membrane preparation of strain Sudlon, saturation binding was not reached and meaningful equilibrium kinetics could not be calculated, thus, for the first time providing clear functional evidence by using radioligand binding assays that the G4946E mutation confers RyR target-site resistance to diamide insecticides (Fig. 2.3). Recent approaches using a fluorescent probe largely failed to provide such compelling evidence for functional implications in diamide binding to RyR´s carrying a G4946E mutation, particularly due to the low specificity of the diamide-like probe showing K_D-values of greater than 1000 nM in fluorescence polarization binding assays (Guo *et al.* 2014a and b). The differences in RyR binding affinity using the fluorescent tracer between diamide resistant and susceptible populations were less than 3-fold, i.e. hardly explaining reported resistance ratios of greater than 1000-fold.

In this study we provided a second line of evidence for G4946E-mediated functional RyR target-site resistance by demonstrating that both flubendiamide and chlorantraniliprole allosterically increased [³H]ryanodine binding in RyR preparations of strain BCS-S at low to sub-nanomolar EC_{50}-values. Thus supporting the general view of diamide activity as a stimulator of the distinct but coupled [³H]ryanodine site. A principal observation recently described by others to be a reliable indicator of diamide action on insect RyR´s (Lümmen *et al.* 2007, Qi *et al.* 2014). As expected from our equilibrium binding studies with [³H]PAD1 the EC_{50}-values for ryanodine binding stimulation in strain Sudlon were at least 100-fold higher for

both chemotypes. The importance of the G4946E mutation is further supported by its presence in diamide resistant diamondback moth larvae we collected in seven different countries other than the Philippines, Thailand and China. We also confirmed the mostly homozygous presence of the G4946E mutation in almost all diamondback populations collected in major vegetable production areas in southern provinces of China such as Guangxi, Guangdong and Fujian. This has also been shown to some extent in recent resistance monitoring initiatives, particularly in Guangdong province where insecticides such as diamides are heavily used (Wang *et al.* 2013, Gong *et al.* 2014). The climate in southern China favours a year round production cycle with as many as 20 generations per year, whereas in northern China such as Shandong province the number of generations and thus selection pressure is much lower (Fig. 2.2, Wang and Wu 2012). All populations we collected in Shandong province were mostly homozygous susceptible, supporting the assumption that the higher the number of generations the more rapidly resistance to diamides develops.

The determination of the genetics of diamide resistance in strain Sudlon revealed almost complete diamide susceptibility of F1 heterozygotes resulting from reciprocal crosses, i.e. a recessive autosomally inherited trait. Our results obtained in back-crossing experiments furthermore suggests that diamide resistance in strain Sudlon is under monogenic control, i.e. strongly supporting that the resistant phenotype is solely based on the G4946E mutation, as no other mutations were found in this strain (Troczka *et al.* 2012). Very recently Guo *et al.* (2014b) described in a diamondback moth population collected in Yunnan province (strain TH) along with G4946E three novel mutations, i.e. E1338D, Q4594L and I4790M. The conservative mutation E1338D is located near the N-terminal end of the *Plutella* RyR, whereas Q4594L and I4790M were linked and located in the C-terminal end. We have shown that RyR G4946E heterozygotes express a diamide susceptible phenotype, thus supporting the assumption of Guo *et al.* (2014b) that the novel mutations are likely to be linked to diamide resistance, as G4946E was only present in 20% of the analysed individuals. In a computational modelling approach we mapped the mutations to a recently published closed-state cryo-EM structure of rabbit RyR1 (Yan *et al.* 2015). The model suggests that the G4946E mutation is located at the interface between helix S4 and the S4–S5 linker, supposed to have a critical role in RyR gating by impacting the movement of pore associated helices (Ramachandran *et al.* 2013). The second mutation I4790M is located in helix S2 in close proximity to G4946E (Fig. 2.5). Furthermore we used the model to determine those amino acid residues within a distance of 7 Å from a virtual midpoint between Cα positions of I4790 and G4946 analogous residues in rabbit PDB 3J8H, which are possibly involved in forming the diamide binding site (Fig. 2.6). The cryo-EM structure of RyR1 represents a closed state, but the close proximity of mutation I4790M to G4946E may suggest functional implications for diamide binding, albeit diamides normally do not bind to closed insect RyR´s (Lümmen *et al.* 2007). The third *Plutella* RyR mutation,

Q4594L described by Guo *et al.* (2014) couldn´t be appropriately mapped due to insufficient resolution at the respective position in the rabbit RyR1 cryo-EM structure. However, the mutation site is not in the trans-membrane domains S1-S6, but in a region with several predicted EF hand domains (Takeshima *et al.* 1989). Our predictions concerning amino acid residues forming a putative diamide binding site in insect RyR is supported by the fact that six out of the 15 suggested amino acids are enclosed in a critical region recently described to confer sensitivity to diamide insecticides in the *Drosophila* RyR (Tao *et al.* 2013). Last but not least it is worth to mention that Q4594 and I4790 are conserved in lepidopteran RyR sequences, but different in other insect orders, whereas G4946 is highly conserved in insects and even vertebrate RyR´s (Fig. 2.6). The lepidoptera specific amino acid residues Q4594 and I4790 are methionine and lysine, respectively in RyR´s of a diverse range of non-lepidoptera species, including diamide sensitive pests such as Colorado potato beetle (Genbank AHW99830.1). Having said this it is tempting to speculate that the Q4594 and I4790 mutation sites identified in the *P. xylostella* RyR are possibly involved in selectivity issues observed for anthranilic and phthalic diamides in different insect species with varying receptor sensitivity (Qi and Casida, 2013, Qi *et al.* 2014). However, site-directed mutagenesis and subsequent functional expression of insect RyR´s will help to study the importance of individual amino acid residues for diamide binding and selectivity in the vicinity of *P. xylostella* (Lepidoptera) RyR G4946 and I4790, thus allowing a refinement of the presented preliminary model considerations in the future.

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Chapter 3

Ryanodine receptor point mutations confer diamide insecticide resistance in tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae)

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Abstract

Insect ryanodine receptors (RyR) are large tetrameric calcium release channels and the molecular target-site for the recently introduced diamide insecticides. Diamides are particularly active on herbivorous pest insects of the order Lepidoptera, such as the tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae), a notorious pest of tomatoes and some other solanaceous crops. High levels of diamide resistance associated with control failure were recently described in some European populations of *T. absoluta*, however, the mechanisms of resistance and their implications for the management of this important pest remained unknown. In this study the molecular basis of diamide resistance was investigated in a diamide resistant strain of *T. absoluta* from Italy (IT-GELA-SD4), which was selected with chlorantraniliprole, and additional resistant field populations collected in Greece, Spain and Brazil. Laboratory selection failed to increase detoxification enzyme levels in the IT-GELA-SD4 strain, however, the activity of diamides was synergized by esterase inhibitors suggesting a possible role for this enzyme family in resistance. Investigation of the genetics of resistance by reciprocally crossing the IT-GELA-SD4 strain with a susceptible strain revealed an autosomal incompletely recessive mode of inheritance. To investigate the role of target-site resistance the gene encoding the RyR in *T. absoluta* was manually curated from an unpublished transcriptome of this species and sequence verified. Pyrosequencing-based genotyping of the IT-GELA-SD4 and field-collected *T. absoluta* strains showing different levels of diamide resistance revealed the presence of the known RyR target-site mutations, G4903E and I4746M. These amino acid substitutions correspond precisely to those recently described for diamide resistant diamondback moth (*Plutella xylostella*), i.e. G4946E and I4790M. We also detected two novel mutations, G4903V and I4746T, in some of the resistant *T. absoluta* strains. Radioligand binding studies with thoracic sarco-/endoplasmic membrane preparations of the IT-GELA-SD4 strain, largely consisting of individuals homozygous for either G4903E, G4903V and/or I4746M, provided functional evidence that these mutations alter the affinity of the RyR to diamides. In combination with previous work on *P. xylostella* our study highlights the importance of position G4903 (G4946in *P. xylostella* RyR) of the insect RyR in defining sensitivity to diamides. The discovery of diamide resistance mutations in *T. absoluta* populations of diverse geographic origin has serious implications for the continued efficacy of this insecticide class for control of this pest, and appropriate resistance management strategies, including mode of action rotation, is strongly advised to delay the further spread of resistance.

3.1 Introduction

The chemical class of diamide insecticides is a relative recent introduction to the market for the control of herbivorous crop pests, particularly lepidopteran larvae (Nauen 2006 and Jeanguenat 2013). The phthalic acid diamide flubendiamide (Tohnishi *et al.* 2005 and Hirooka *et al.* 2007) was introduced in 2006, followed by the anthranilic diamides chlorantraniliprole and cyantraniliprole (Lahm *et al.* 2007 and Lahm *et al.* 2009). The global turn-over of the whole chemical class was >\$1.2 billion in 2013, representing approx. 8% of the insecticide market (Sparks and Nauen 2015). Both flubendiamide and chlorantraniliprole are highly active against a broad range of lepidopteran pests at low application rates, show low acute mammalian toxicity, a favorable environmental profile, and can be used in integrated pest management programmes (Tohnishi *et al.* 2005, Lahm *et al.* 2007).

Diamide insecticides act as conformation sensitive activators of the insect ryanodine receptor (RyR), a large (homo)tetrameric calcium-channel located in the sarco- and endoplasmic reticulum in neuromuscular tissues (Ebbinghaus-Kintscher *et al.* 2006, Cordova *et al.* 2006, Lümmen *et al.* 2007, Sattelle *et al.* 2008). The endogenous activation of RyRs is mediated by calcium influx, driven by voltage-gated calcium channels upon depolarization of the cell membrane (Lümmen 2013). The symptomology of poisoning after diamide application involves muscle contraction, paralysis and eventually death (Tohnishi *et al.* 2005, Cordova *et al.* 2006). In contrast to mammals which possess three *RyR* genes (Rossi and Sorrentino 2002), insects encode a single *RyR* gene with an open reading frame (ORF) of >15,000 nucleotides translated into a RyR protomer with a molecular weight of more than 5000 kDa, as first described for *Drosophila melanogaster* (Takeshima *et al.* 1994). RyRs were shown to be composed of six helical transmembrane spanning domains at the C-terminal end containing the calcium ion-conducting pore, and a large N-terminal cytosolic domain (Lümmen 2013). Recently Yan *et al.* (2015) published a rabbit RyR1 structure determined by single-particle electron cryomicroscopy which resolved a large portion of the homotetrameric channel protein (Yan *et al.* 2015).

Biochemical studies with a series of *Bombyx mori* RyR deletion mutants suggested that the diamide binding site is likely to be located in the C-terminal transmembrane spanning domain (Kato *et al.* 2009). This was further supported by the identification of target-site mutations, G4946E and I4790M, in the RyR transmembrane domain of diamide resistant strains of diamondback moth *Plutella xylostella* (Troczka *et al.* 2012 and Guo *et al.* 2014), and functionally confirmed by radioligand binding studies (Steinbach *et al.* 2015), and fluorescence based reporter assays using Sf9 cells stably expressing a modified (G4946E) diamondback moth RyR (Troczka *et al.* 2015).

Diamide insecticides are used globally straight and in mixtures for foliar, drench and seed treatment applications in a broad range of agricultural and horticultural cropping systems. The popularity and widespread uptake of diamides has increased selection pressure for the evolution

of resistance, particularly in the case of lepidopteran pests (Teixeira and Andaloro 2013). The global status of diamide resistance issues in lepidopteran pests was recently reviewed by Nauen and Steinbach (2016). High levels of resistance compromising the efficacy of diamides at recommended field rates were reported in very few species. The first serious cases of resistance were described for diamondback moth strains collected in the Philippines and Thailand (Troczka *et al.* 2012), followed by China (Wang and Wu 2012, Wang *et al.* 2013, Gong *et al.* 2014), Brazil (Ribeiro *et al.* 2014), Taiwan, India, USA, Japan, Korea and Vietnam (Steinbach *et al.* 2015). The underlying basis of resistance in a number of field-collected diamondback moth strains appears to be largely due to target-site mutations in the transmembrane domain of the RyR (Troczka *et al.* 2012, Guo *et al.* 2014, Steinbach *et al.* 2015). In particular the G4946E mutation - located at the interface between helix S4 and the S4–S5 linker - was functionally linked to high levels of diamide resistance (Steinbach *et al.* 2015, Troczka *et al.* 2015). The functional significance of other mutation sites such as I4790M and Q4594L, present in recently collected Chinese *P. xylostella* populations, is less clear (Guo *et al.* 2014). Nevertheless, homology modeling of the *Plutella* RyR based on the structure of rabbit RyR1 (Yan *et al.* 2015), revealed that the I4790M mutation in helix S2 is in quite close proximity to G4946E, suggesting a potential functional role in diamide binding (Steinbach *et al.* 2015). Apart from diamondback moth, high levels of diamide cross-resistance under applied conditions were only described in two other lepidopteran pest species, the smaller tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014) and the tomato leafminer, *Tuta absoluta* (Meyrick) (Roditakis *et al.* 2015, Silva *et al.* 2016).

Tuta absoluta is a multivoltine, invasive pest that originated from Latin America and has recently spread into Europe, Africa and the Middle East, threatening tomato production in both open field and greenhouse crops (Desneux *et al.* 2010). Recent investigations have shown that *T. absoluta,* which first invaded the Mediterranean Basin in 2006, most likely originates from Chile (Guillemaud *et al.* 2015). Given its invasive nature and destructive potential it has quickly gained status as a global pest of key concern (Desneux *et al.* 2011). *Tuta absoluta* control relies heavily on insecticide treatments (Roditakis *et al.* 2013), however, reports of control failure have clearly illustrated the potential of this pest to develop resistance to multiple classes of insecticide (Siqueira *et al.* 2000, Siqueira *et al.* 2001, Silva *et al.* 2011, Silva *et al.* 2016 and Haddi *et al.* 2012; J.E. Silva *et al.* 2016). Baseline susceptibility studies showed diamide insecticides are highly effective against *T. absoluta* (Roditakis *et al.* 2013, Campos *et al.* 2015). However, recently, *T. absoluta* collected from Italian greenhouse tomatoes exhibited resistance to diamides of more than 1000-fold when compared to a susceptible reference strain (Roditakis *et al.* 2015). Despite the reliance on diamides for control of *T. absoluta* in many countries, the molecular mechanisms conferring diamide resistance in *T. absoluta* and implications for resistance management remain unknown. In the present study we investigated

the molecular basis of diamide resistance in *T. absoluta* populations collected in both Europe as well as South America and describe target-site resistance to this insecticide class in *T. absoluta* for the first time.

3.2 Material and methods

3.2.1 Insect strains

The *T. absoluta* field strains used in this study were collected from infested tomato (*Solanum lycopersicum* L.) crops in Italy, Greece, Spain and Brazil between 2014 and 2015. A number of tomato leaves infested with *T. absoluta* larvae were collected in large plastic bags and transferred to the respective laboratory for experimentation. Details for each collected strain are provided in Table 3.1. At least 300 individuals were collected in each location and the larvae were allowed to develop on 3–6 week old potted tomato plants. All populations were maintained in insect cages at 26 ± 1 °C, 65% RH and 16 h light: 8 h dark photoperiod. The susceptible reference strains (GR-Lab, ES-Sus and BCS-TA-S) were maintained for several years under laboratory conditions without any exposure to insecticides. Strain IT-GELA-SD4 was obtained after four sequential selection cycles of strain IT-GELA-14-1 with the insecticide chlorantraniliprole using foliar applied doses of 100 mg L^{-1} (first selection) and 300 mg L^{-1} (subsequent selections). At least 1000 2nd instar larvae were used for each selection cycle.

Table 3.1. Information on field and laboratory strains of *Tuta absoluta* used in the present study. All strains were collected and/or maintained on tomato plants. Strain IT-GELA-SD4 was obtained from strain IT-GELA-14-1 after 4 selection cycles with chlorantraniliprole (see M&M).

	Strain	Country	Location	Year
Reference lab	GR-Lab	Greece	Peloponnese	2010
	ES-Sus	Spain	Murcia, Aguilas	2011
	BCS-TA-S	Brazil	Paulinia, SP	2005
Field-collected	GR-IER-15-2	Greece	Ierapetra, Kalogeri	2015
	IT-GELA-14-1	Italy	Sicily, Gela	2014
	ES-MUR-14	Spain	Murcia, Lorca	2014
	BR-GML1	Brazil	Gameleira, BA	2014
	BR-PSQ	Brazil	Pesqueira, PE	2014
Selected	IT-GELA-SD4	Italy	Sicily , Gela	2014

3.2.2. Chemicals and insecticides

All chemicals and organic solvents used were of analytical grade. For all bioassays and insecticide treatments commercial formulations of the diamide insecticides chlorantraniliprole (Altacor® 35WG, DuPont, France) and flubendiamide (Belt® 24WG, Bayer CropScience AG, Germany) were used. For Brazilian strains another commercial formulation of chlorantraniliprole was used (Premio® 200SC, DuPont, Brazil).

3.2.3. Insect bioassays

Leaf dip bioassays with the European populations were principally conducted according to IRAC method 022 (www.irac-online.org) with slight modifications described elsewhere (Roditakis *et al.* 2013). Briefly, either tomato leaflets cut in square pieces, or entire leaves were immersed in serial insecticide concentrations containing Triton X-100 (0.2 g L^{-1}) as a non-ionic wetting agent. Treated leaves were allowed to dry for 1–2 h at room temperature and subsequently placed adaxially on moist tissue paper in a multi-well repli-dish. A single 2nd instar larva was placed in each well, subsequently all wells were sealed with transparent ventilated adhesive lids. Bioassays with the Brazilian field strains were conducted with a slightly different method according to Campos *et al.* (2015). All bioassays were incubated in growth chambers at 25 ± 0.5 °C and $65 \pm 5\%$ relative humidity. Larval mortality was assessed after 3 days of exposure. Mortality evaluations were performed with the aid of a light source and magnifying glass. Larvae were carefully removed from tomato leaf galleries and considered dead if they were unable to move the length of their bodies after gentle prodding with a camel-hair brush.

Lethal concentration values for 50% mortality (LC$_{50}$) and 95% confidence limits (CL 95%) were obtained by probit analysis using the software packages PriProbit 3.4 (Sakuma 1998) or Polo Plus (LeOra software, USA). LC$_{50}$-values were considered significantly different when their 95% confidence limits did not overlap. Percentage mortality values generated in bioassays was corrected using Abbott's formula (Abbott 1925).

3.2.4. Synergist bioassays

Synergist bioassays were performed following IRAC method 022 as described above with strain IT-GELA-SD4 (selected with chlorantraniliprole). Two hours prior to bioassay, larvae were exposed to sublethal doses ($\leq$LC$_{05}$) of different synergists via contact on fresh dried residues in coated glass vials (30 ml volume). Synergists used were piperonyl butoxide (PBO, Sigma, UK), S,S,S tributyl phosphorotrithioate (DEF, Sigma, UK) and diethyl maleate (DEM, Sigma, UK), known to inhibit cytochrome P450 monooxygenases, esterases and glutathione S-transferases, respectively. For vial coating 300 µl of acetonic solutions of PBO (0.1 g L^{-1}), DEF (0.1 g L^{-1}) or DEM (0.3 g L^{-1}) were added into each vial. Afterwards vials were placed horizontally on rotating metal rods for 1 h. After rotation the vials were allowed to dry for another hour before adding the larvae. The synergistic ratio was calculated by dividing the calculated LC$_{50}$-value of synergist-exposed larvae by the LC$_{50}$-value of larvae not exposed to synergists.

3.2.5. Genetics of diamide resistance

Tuta absoluta larvae of strains GR-Lab and IT-GELA-SD4 were allowed to develop and pupate under controlled conditions (see above) in small plastic rearing cages. Pupae were subsequently collected and sexes separated based on external morphology (Solomon 1962). Subsequently they were placed individually in transparent glass tubes where the emergence of adults was checked daily. The sex of adult moths was checked again for confirmation of the initial classification of pupae (Coelho and França 1987). Subsequently 100 virgin females of strain IT-GELA-SD4 were crossed with 100 males of the susceptible strain GR-Lab and *vice versa*. The strains were considered to have similar genetic background due to the minimal genetic variability observed among the European *T. absoluta* strains (Cifuentes *et al.* 2011 and Guillemaud *et al.* 2015). Second instar larvae of the F1 generation of reciprocal crosses were subsequently bioassayed with chlorantraniliprole to determine LC_{50}-values as described above. The degree of dominance was calculated using the formula $D = (2X_2-X_1-X_3)/(X_1-X_3)$ (Stone 1968). The values X_1, X_2 and X_3 are the log (LC_{50}) of the resistant strain IT-GELA-SD4, the F1 generation and the susceptible strain GR-Lab, respectively. The F1 generation of the reciprocal crosses was subsequently back-crossed with the parental IT-GELA-SD4 strain to check for monogenic resistance. Expected mortality of the backcross generation (F2) under a monogenic model was calculated using the following formula: expected % mortality F2 at [c] = 0.5 × (% mortality of F1 at [c] + % mortality of strain IT-GELA-SD4 at [c]), where [c] refers to the respective concentration of chlorantraniliprole.

3.2.6. Biochemical assays

All enzymatic assays were repeated at least three times. Detoxification enzyme activities were determined in strains GR-Lab (susceptible), IT-GELA-14-1 (field-collected) and IT-GELA-SD4 (selected). Glutathione S-transferase (GST), esterase (EST) and cytochrome P450 monooxygenase (P450) activities were determined using a SpectraMax M2e microplate reader (Molecular Devices, Berkshire, UK). For GST and EST activities, groups of five 3rd instar larvae were homogenized on ice in 100 μl of 0.1 M sodium phosphate buffer, pH 7.2 and centrifuged for 5 min at 4 °C and 5000 g. The supernatants were used as enzyme source. Total protein content of the enzyme solution was determined by the Bradford method using bovine serum albumin as the standard (Bradford 1976).

For GST activity, the homogenate (10–20 μg of total protein) was mixed with 200 μL of substrate solution (10 mM reduced glutathione, 3 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 100 mM sodium phosphate buffer, pH 6.5). The activity was measured kinetically at 340 nm for 5 min and expressed as μmol mg protein^{-1} min^{-1} using the extinction coefficient of the resulting 2,4-dinitrophenylglutathione conjugate at 340 nm (9.6 mM^{-1} cm^{-1}).

EST activities were determined using 1- and 2-naphthyl acetate (NA) as substrate. Homogenate (2–5 µg of total protein) was placed in a microplate well, and 200 µl naphthyl acetate working solution was added (0.3 mM 1-NA or 2-NA in sodium phosphate buffer 0.2 M, pH 7.2). After 30 min incubation, 50 µl of 6.4 mM Fast Blue B salt (Sigma Aldrich) diluted in 35 mM sodium phosphate buffer pH 7, containing 35 g L^{-1} SDS were added to each well. The formation of the 1- or 2-naphthol Fast Blue dye complex was measured at 570 nm and converted to specific activity expressed as 1- or 2-naphthol mg protein-1 min-1, using a standard curve of 1- or 2-naphthol. Standard curves were established to determine the quantity of formed final product.

P450 activity was determined by the O-deethylation of 7-ethoxycoumarin (7-EC) using a protocol adapted for *in vivo* analysis in microplates (Reyes *et al.* 2012). Briefly twenty 3rd instar larvae were dissected individually placed in the wells of black microplates. Each well contained 0.4 mM 7-EC in 0.1 ml of 50 mM HEPES buffer, pH 7.0. Ten wells without dissected insects were used as controls. After 4 h incubation at 30 °C, the enzymatic reaction was stopped by adding 0.1 ml 0.1 mM glycine buffer (pH 10.4):ethanol (v/v). Umbelliferone fluorescence was measured at 465 nm while exciting at 390 nm. A standard curve was established using umbelliferone and cytochrome P450 activity was expressed as pg product insect^{-1} min^{-1}.

3.2.7. Radioligand binding studies

Tuta absoluta adults of strains BCS-TA-S and IT-GELA-SD4 were flash-frozen in liquid nitrogen, stored at −80 °C and collected over many generations to get the appropriate biomass necessary for conducting radioligand binding assays. Thoracic endoplasmic/sarcoplasmic reticulum membranes were prepared as described earlier (Steinbach *et al.* 2015). Radioligand binding assays were performed using 50 µg membrane protein per assay. Enhancement of ryanodine binding was measured using 4.0 nM [^{3}H]ryanodine as a function of increased diamide insecticide concentration as described elsewhere (Steinbach *et al.* 2015). Equilibrium binding parameters were calculated using the software package GraphPad Prism 5 (GraphPad Inc.).

3.2.8. Identification and sequence verification of the *T. absoluta* RyR

The *T. absoluta* RyR was manually curated from an unpublished transcriptome of this species, generated by Illumina sequencing of different life stages of a diamide susceptible strain, followed by *de novo* assembly using Trinity (Grabherr *et al.* 2011). To verify the transcriptome sequence 14 primer pairs were designed (Appendix A: Table S1), spanning the entire ORF. RNA was extracted from pools of 10–20 larvae using the ISOLATE II RNA Mini Kit (Bioline) and quantified using a NanoDrop® 1000 (ThermoScientific, USA). 5 µg was used for cDNA synthesis using SuperScript III RT (ThermoScientific, USA) and random hexamers (Promega, USA). PCR reactions (25 µl total volume) contained Dreamtaq mastermix (ThermoScientific,

USA) 1 μl of cDNA and 10 pmol of each primer pair. Cycling conditions were 95 °C for 2min followed by 35 cycles of 95 °C for 20s, 50 °C for 20s and 72 °C for 2 min, with a final extension step of 72 °C for 5min. PCR products were visualised on a 1 % TAE agarose gel electrophoresis and purified via QIAquick PCR purification kit (Qiagen, Germany). All PCR products were sequenced using the Sanger method by Eurofins (Germany). Sequencing results were analyzed using Geneious software (Biomatters, New Zeland). The final curated sequence was deposited in NCBI under GenBank accession no. KX519762.

3.2.9. Pyrosequencing of PCR amplified RyR cDNA of *T. absoluta* for genotyping

Individuals of different strains of *T. absoluta* were genotyped for the recently described diamondback moth RyR mutations G4946E (Troczka *et al.* 2012, Steinbach *et al.* 2015) and I4790M (Guo *et al.* 2014). Genomic DNA was extracted from individual larvae using the DNAdvance Tissue Kit (Agencourt) according to the supplier's recommended protocol. Primer pairs were designed based on the obtained RyR full-length cDNA sequence (GenBank no. KX519762). A short gene fragment of 228 bp and 190 bp for G4946E and I4790M genotyping, respectively was amplified by PCR from 50 ng aliquots of *T. absoluta* gDNA using the primer pairs Ta_I4790__F, Ta_I4790-R-btn and Ta_G4946-F-btn, Ta_G4946-R, respectively (Appendix A: Table S2). The pyrosequencing protocol for the detection of I4790M (position 4746 in *Tuta* RyR) comprised 40 PCR cycles with 0.5 μM forward and biotinylated reverse primer in 30 μl reaction mixtures containing 1 × Taq enzyme reaction mix (JumpStart™ Taq ReadyMix™, Sigma Aldrich) and cycling conditions of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. The same protocol was used for genotyping G4946E (position 4903 in *Tuta* RyR) with a few modifications: the forward primer was biotinylated rather than the reverse primer and the temperature for primer annealing was set to 53 °C for 30 s. The single strand DNA required for pyrosequencing was prepared as described in Troczka *et al.* (2012). The pyrosequencing reactions were carried out according to the manufacturer's instructions using the PSQ 96 Gold Reagent Kit (Qiagen), and the sequencing primer Ta_I4790-Seq-F (I4790M) or Ta_G4946-Seq-R (G4946E) for genotyping. The pyrograms, indicating the genotype, were analyzed using the SNP Software (Qiagen).

3.2.10. Sanger sequencing

Sanger sequencing was performed in order to partially sequence the RyR domain of Brazilian strains BR-GML1 and BR-PSQ that failed to produce results in G4946E-pyrosequencing diagnostics. Degenerate primers, Ta_SangerSeq-F and Ta_SangerSeq-R (Appendix A: Table S2), were designed based on a multiple nucleotide alignment of *T. absoluta* (GenBank no.

KX519762), *P. xylostella* (JN801028), *B. mori* (XM_004924859) and *Chilo suppressalis* (JX082287). A 285 bp fragment of *T. absoluta* RyR was amplified from gDNA by PCR using Q5® High-Fidelity DNA Polymerase 2× Master Mix according to the manufacturer's instruction (New England BioLabs Inc., USA). The cycling conditions were 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 50 °C for 30 s and 72 °C for 30 s, and a final elongation step at 72 °C for 2 min. The purified PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany).

3.3 Results

3.3.1. Leaf-dip bioassays and stability of resistance

Three diamide susceptible laboratory reference strains, five field-collected strains from Italy, Greece, Spain and Brazil, and one chlorantraniliprole-selected strain originating from Italy (IT-GELA-SD4) were included in this study. The obtained LC_{50}-values for chlorantraniliprole in the susceptible reference strains GR-Lab, ES-Sus and BCS-TA-S differed less than 2-fold (between 0.18 and 0.31 mg L^{-1}; Table 3.2). Resistance ratios for chlorantraniliprole in field collected strains varied between 8 (strain ES-MUR-14, Spain) and >3000 (strain BR-PSQ, Brazil). Strain IT-GELA-SD4 selected with chlorantraniliprole for four generations exhibited a resistance ratio 4-fold higher than the parental strain IT-GELA-14-1 (Table 3.2). Strain IT-GELA-SD4 was mass reared and chosen for more detailed genetic and molecular studies on diamide resistance in *T. absoluta*. Whereas parental strain IT-GELA-14-1 lost some of its resistance over time, we observed a stable diamide resistance of selected strain IT-GELA-SD4 over a period of 10 months (data not shown).

Table 3.2. Log-dose probit-mortality data for chlorantraniliprole against 2nd instar larvae of different strains of *Tuta absoluta* in foliar bioassays (96h).

	Strain	N	LC$_{50}$ [mg L^{-1}]	CL 95%[d]	Slope	RR
Reference	GR-Lab	192	0.31	0.22-0.45	1.6	
	ES-Sus	300	0.18	0.14-0.23	2.3	
	BCS-TA-S	210	0.21	0.15-0.29	1.4	
Field	GR-IER-15-2	145	17	8.7-42		55[a]
	IT-GELA-14-1	191	56	14-120	1.0	181[a]
	ES-MUR-14	210	1.5	1.1-2.1	1.6	8[b]
	BR-GML1	296	92	60-130	1.2	438[c]
	BR-PSQ	292	650	420-920	1.2	3095[c]
Selected	IT-GELA-SD4	127	230	110-430	1.2	742[a]

[a] Resistance ratio (RR) calculated is based on strain GR-Lab

[b] Resistance ratio (RR) calculated is based on strain ES-Sus

[c] Resistance ratio (RR) calculated is based on strain BCS-TA-S

[d] Confidence limits 95%

3.3.2. Genetics of resistance of strain IT-GELA-SD4

Results of the bioassays with chlorantraniliprole against strains GR-Lab (S) and IT-GELA-SD4 (R), as well as their reciprocal crosses are presented in Table 3.3. Although the LC$_{50}$-values obtained in bioassays with the respective F1 generation of reciprocal crosses were 3-fold different, such marginal differentiations could be more likely caused by bioassay variably factors rather than sex linkage. Therefore, negligible maternal effect was observed suggesting that diamide resistance in *T. absoluta* is autosomal inherited. The calculated degree of dominance (D) was −0.63 and −0.29 for (S)♀ × (R)♂ and (S)♂ x (R)♀, respectively, suggesting an incompletely recessive mode of inheritance. Female F1 hybrids of (S)♀ x (R)♂ crosses were backcrossed with males of IT-GELA-SD4 and tested for monogenic resistance. However the obtained experimental dose-response curve for chlorantraniliprole did not clearly plateau at 50 % mortality and was significantly different from theoretical considerations assuming monogenic inheritance ($X^2 = 28.3$, df = 8, $P < 0.05$), possibly indicating that diamide resistance in strain IT-GELA-SD4 is not controlled by a single trait (Fig. 3.1).

Table 3.3. Log-dose probit-mortality data for chlorantraniliprole tested against reciprocal crosses of 2nd instar larvae of diamide susceptible (S) and resistant (R) *Tuta absoluta* strains GR-Lab and IT-GELA-SD4, respectively.

Strain	N	LC$_{50}$ [mg L^{-1}]	CL 95%[a]	Slope	D[b]
GR-Lab (S)	192	0.36	0.23-0.54	1.4	

IT-GELA-SD4 (R)	189	185		111-288	1.2	
F1: (S)♀ x (R)♂	145	1.1		0.67-1.8	1.4	-0.64
F1: (S)♂ x (R)♀	208	3.3		1.9-5.1	1.4	-0.29

[a] Confidence limits 95%

[b] Degree of dominance
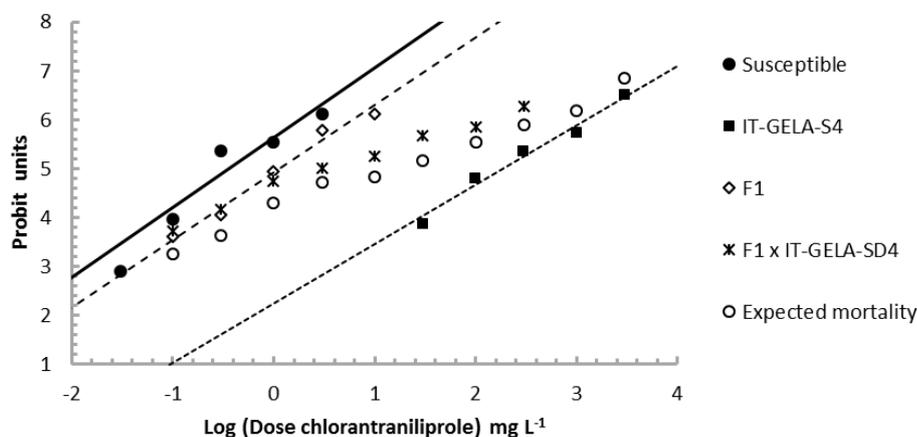
Figure 3.1. Log-dose probit-mortality lines for chlorantraniliprole tested against 2nd instar larvae of *Tuta absoluta*. Probit lines for strains GR-LAB (susceptible), IT-GELA-SD4, F1-a hybrids (GR-LAB♀ x IT-GELA-SD4♂), F1-a♀ x IT-GELA-SD4♂ backcross and its calculated theoretical backcross based on monogenic resistance. Data obtained for F1 hybrids resulting from GR-LAB♂ x IT-GELA-SD4♀ crosses are not shown as they were not significantly different.

3.3.3. Diamide cross-resistance, effect of synergists and activity levels of detoxification enzymes in strain IT-GELA-SD4

Strain IT-GELA-SD4 showed strong cross-resistance to the phthalic acid diamide flubendiamide (RR > 2500-fold), demonstrating the presence of a resistance mechanism affecting both anthranilic and phthalic acid diamides (Table 3.4). In order to investigate the possible involvement of a metabolic mechanism of resistance we tested the effect of synergists on the efficacy of both chlorantraniliprole and flubendiamide against strain IT-GELA-SD4. Glutathione depletion by DEM and inhibition of cytochrome P450 monooxygenases by PBO prior to diamide exposure resulted in synergistic ratios less than or equal to 2-fold, suggestive of a rather limited impact of these detoxification mechanisms in strain IT-GELA-SD4. In bioassays with the esterase inhibitor DEF combined with chlorantraniliprole we observed a synergistic ratio of 11-fold compared to chlorantraniliprole alone, suggesting the possible presence of an esterase-mediated mechanism of resistance for this particular insecticide only (Table 3.4), since comparable synergistic action with DEF was not observed when flubendiamide was tested. However, the chlorantraniliprole resistance ratio in strain IT-GELA-SD4 was still >100-fold in DEF synergised bioassays when compared to the susceptible reference strain GR-Lab, clearly indicating the presence of additional mechanisms of resistance. The activity levels of detoxification enzyme families measured with artificial model substrates

were similar in larvae of strains GR-Lab, IT-GELA-14-1 and IT-GELA-SD4 (Table 3.5), with no significant increase in activity observed in GSTs, CEs, or P450s in strain IT-GELA_SD4 compared with the GR-Lab susceptible strain.

Table 3.4. Log-dose probit-mortality data for chlorantraniliprole (CPR) and flubendiamide (FLB) tested in combination with synergists against 2nd instar larvae of *Tuta absoluta*.

Strain	N	LC$_{50}$ [mg L^{-1}]	CL 95%[a]	Slope	RR	SR[b]
CPR						
GR-Lab	187	0.18	0.13–0.30	1.4		
IT-GELA-SD4	92	244	29–410	2.5	1356	
IT-GELA-SD4 +DEF	168	23	14–35	1.7	128	11
IT-GELA-SD4 +PBO	100	161	82–430	1.4	894	2
IT-GELA-SD4 +DEM	95	169	80–1000	1.0	939	1
FLB						
GR-Lab	186	0.79	0.31–1.5	1.1		
IT-GELA-SD4	160	2100	1300–4300	1.4	2658	
IT-GELA-SD4 +DEF	100	1255	496–16,035	0.87	1589	2
IT-GELA-SD4 +PBO	116	1284	626–3520	0.78	1625	2
IT-GELA-SD4 +DEM	112	1700	550–5700	1.3	2152	1

[a] Confidence limits 95%

[b] Synergistic ratio (LC$_{50}$ without synergist divided by LC$_{50}$ + synergist)

Table 3.5. Detoxification enzyme activity measured with model substrates for esterases (EST), glutathione S-transferases (GST) and microsomal monooxygenases (P450) in different strains of *Tuta absoluta*. Results are mean values ± SD (n=3) with no statistically significant differences between strains.

Strain	EST 1-NA[a]	EST 2-NA[b]	GST CDNB[c]	P450 7-EC[d]
GR-Lab	122 ± 10	265 ± 13	0.258 ± 0.058	16.0 ± 2.65
IT-GELA-14-1	166 ± 26	251 ± 6.7	0.198 ± 0.036	20.1 ± 2.45
IT-GELA-SD4	141 ± 4.1	249 ± 2.9	0.217 ± 0.022	20.9 ± 2.11

[a] nmol 1-naphthol/min x mg protein (1-NA = 1-naphthylacetate)

[b] nmol 2-naphthol/min x mg protein (2-NA = 2-naphthylacetate)

[c] μmol 2,4-dinitrophenyl-S-glutathione/min x mg protein (CDNB = 1-Chloro-2,4-dinitrobenzene)

[d] pg 7-OH-coumarin/min x larva (7-EC = 7-Ethoxycoumarin)

3.3.4. Radioligand binding studies

Comparative binding studies using thoracic membrane preparations of strains BCS-TA-S and IT-GELA-SD4 were carried out with the diamide insecticide flubendiamide, known to act, like chlorantraniliprole, as a positive allosteric activator of [^{3}H]ryanodine binding to lepidopteran RyRs (Steinbach *et al.* 2015). Strain IT-GELA-SD4 shows high levels of resistance (≥1000-fold) against both flubendiamide and chlorantraniliprole when compared to the susceptible reference strains in this study (Table 3.4). Radioligand binding studies using thoracic endo-/sarcoplasmic membranes of adults of strain BCS-TA-S and IT-GELA-SD4 revealed a relative

increase of [^{3}H]ryanodine binding as a function of flubendiamide concentration at an EC$_{50}$ of 3.03 ± 1.45 nM and >1000 nM, respectively (Fig. 3.2). This equates to RyR target-site insensitivity of >300-fold to flubendiamide in membrane preparations of strain IT-GELA-SD4 when compared to membranes isolated from the diamide susceptible strain BCS-TA-S.
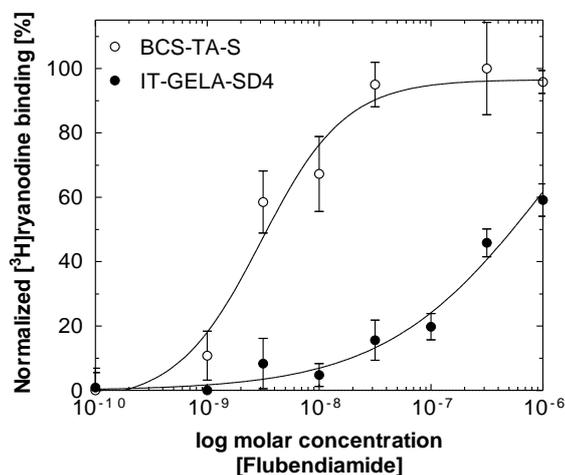
Figure 3.2. Radioligand binding assays using thoracic microsomal membrane preparations of diamide susceptible (BCS-TA-S) and resistant (IT-GELA-SD4) *Tuta absoluta* strains. Relative increase of [^{3}H]-ryanodine binding as a function of diamide insecticide concentration reveal functional implications of the detected G4946E/V and I4790M RyR mutation in strain IT-GELA-SD4 for diamide binding. Data are mean values $\pm$ SD (n=4).

3.3.5. Cloning, sequencing and characterisation of the *T. absoluta* RyR

A single contig of 16,431 bp encoding the *T. absoluta* RyR was identified in our unpublished transcriptome of this species based on BLAST annotation of the whole transcriptome against the non-redundant protein database of NCBI. This transcript included the full length coding sequence of the RyR gene of 15,363 bp encoding 5121 amino acids and shows high levels of sequence similarity with the RyR mRNA of other Lepidoptera (Table 3.6). The complete coding sequence of this contig was independently sequence verified by PCR and the final curated sequence was deposited in NCBI under GenBank accession no. KX519762. The predicted protomer has a molecular mass of 578.965 kDa and shares features common to other characterized insect RyRs, including a large extracellular N-terminal region followed by a highly conserved transmembrane region comprising 6 predicted transmembrane S1—S6 (Fig. 3.3) toward the COOH terminus of the sequence with the probable pore-forming domain located between domains S5 and S6. Several putative resistance hot-spots have been described in the RyR of resistant *P. xylostella* (G4946E, E1338D, Q4594L and I4790M) two of which (G4946E and I4790M) have been most strongly implicated in resistance as detailed in the introduction. The RyR sequence of the diamide susceptible *T. absoluta* strain sequenced had the

'susceptible' amino acid at position 4903 (corresponding to G4946 in *P. xylostella*), 4746 (I4790 in *P. xylostella*), 4540 (Q4594 in *P. xylostella*) but an aspartic acid (D) at position 1339 (E1338 in *P. xylostella*), suggesting the E1338D substitution first reported in *P. xylostella* is not a *bona fide* resistance mutation.

Table 3.6. Comparison of insect ryanodine receptor protein sequences (given in % identity).

	T. absoluta	*C. medinalis*	*H. armigera*	*P. xylostella*	*T. castaneum*	*D. melanogaster*	*A. pisum*
T. absoluta	---						
C. medinalis	93.1	---					
H. armigera	92.7	94.3	---				
P. xylostella	90.7	92.2	92.2	---			
T. castaneum	81.8	82.6	82.0	81.0	---		
D. melanogaster	77.9	78.6	78.3	77.5	77.7	---	
A. pisum	77.3	77.9	77.2	76.5	79.1	74.5	---
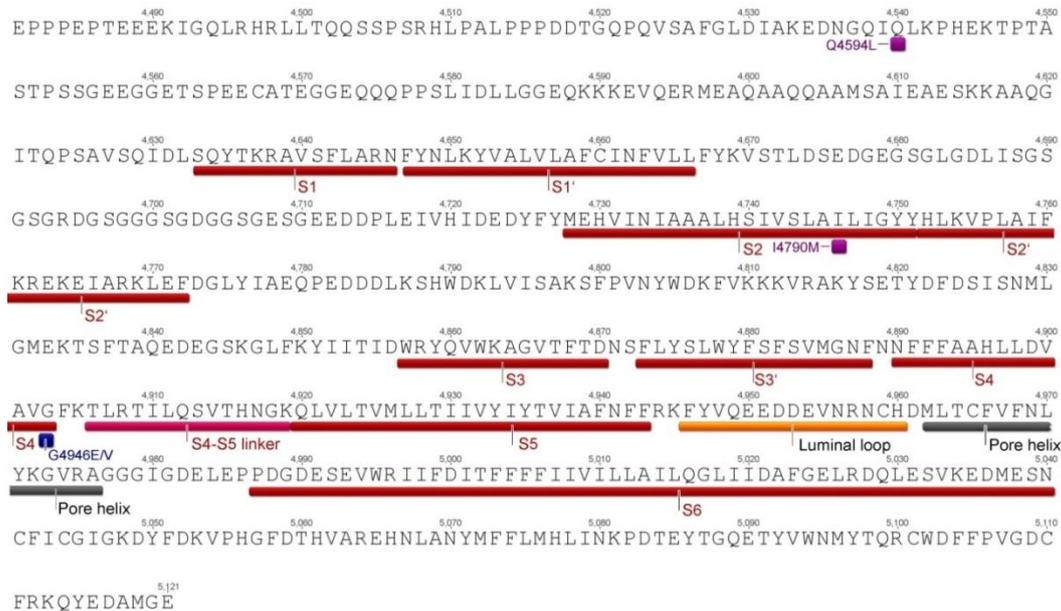
Figure 3.3. Partial amino acid sequence of the C-terminal transmembrane domain of the RyR from *Tuta absoluta*. The full length nucleotide and deduced amino acid sequence has been submitted to GenBank (accession no. KX519762). The positions of the transmembrane-spanning domains S1 to S6 are designated with red bars and based on those recently predicted for the diamondback moth RyR (Steinbach *et al.* 2015). The highly conserved pore helix and the luminal loop are designated with grey and orange bars, respectively. The S4–S5 linker region (marked in magenta) is close to the amino acid residue G4903, which corresponds to the mutation site recently shown to confer diamide resistance in diamondback moth (G4946E). Two further mutation sites, I4790M and Q4594L recently linked to diamide resistance in diamondback moth correspond to amino acid residues I4746 and Q4540,

respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.6. Genotyping for RyR target-site mutations by pyrosequencing

Individuals of a number of strains included in this study were analyzed for the presence and frequency of mutations at positions G4903 and I4746, previously associated with RyR target-site resistance against diamides in *P. xylostella* at the corresponding positions (Steinbach *et al.* 2015, Guo *et al.* 2014). A partial amino acid sequence of the *T. absoluta* RyR C-terminal transmembrane domain indicating potential mutation sites (based on diamide resistant *P. xylostella* RyR) is shown in Fig. 3.3. The pyrosequencing assay using genomic DNA of *T. absoluta* worked well for all strains and both mutation sites (Appendix A: Figs. S1 and S2), except for G4903 of the two Brazilian field strains BR-GML1 and BR-PSQ, due to their polymorphism in the nucleotide sequence of the *RyR* regions selected for primer design as revealed by Sanger sequencing (Appendix A: Fig. S3). However Sanger sequencing of partial *RyR* sequences of both Brazilian field strains highly resistant to diamides revealed the presence of the G4903E mutation (Fig. 3.4) at a frequency of 100 %, albeit only a relatively small number of samples was analyzed (Table 3.7). Furthermore, pyrosequencing of DNA samples of these strains confirmed the presence of the mutations I4746M, and a previously un-reported amino acid substitution, I4746T, at low frequency (Table 3.7). The diamide susceptible strains BCS-TA-S, GR-Lab and ES-Sus were all wildtype homozygous at positions G4903 and I4746 (Table 3.7). The laboratory selected strain IT-GELA-SD4 was found to contain a mixture of individuals of different genotypes none of which had the wildtype G4903 amino acid residue, but rather were homozygous for G4903E, or G4903V, a novel substitution not yet reported at this position. In addition the IT-GELA-SD4 strain also carried the I4746M mutation at high frequency (80 % of individuals tested were homozygous for this mutation). Strain GR-IER-15-2 which exhibited 55-fold resistance to chlorantraniliprole exhibited 10 % and 20 % of sequenced individuals homozygous for G4903E and G4903V, respectively. Most individuals were wildtype I4746, though we detected a low frequency of I4746M heterozygotes.
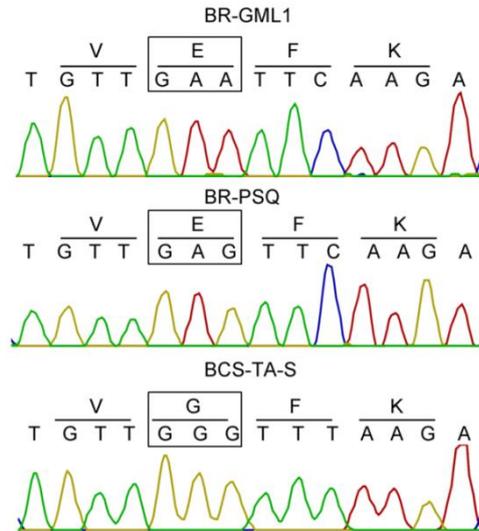
Figure 3.4. Sanger sequencing revealed mutations in the *RyR* gene in Brazilian *Tuta absoluta* field strains. The lower nucleotide sequence obtained from a diamide susceptible laboratory strain (BCS-TA-S) shows the reference sequence GGG (boxed) coding for a glycine at position 4903 in the *T. absoluta* RyR. The upper chromatograms and corresponding sequences clearly show the presence of different single nucleotide polymorphisms (boxed) in diamide-resistant Brazilian field strains BR-GML1 (GAA) and BR-PSQ (GAG), resulting in the amino acid substitution G4903E, which corresponds to the mutation site recently shown to confer diamide target-site resistance in diamondback moth (G4946E).

Table 3.7. Genotyping by pyrosequencing of individuals of *Tuta absoluta* (n = 8–12) for the presence of RyR mutations in the C-terminal transmembrane domain at amino acid positions G4903 and I4746 corresponding to G4946 and I4790 in diamondback moth. All three diamide susceptible strains (BCS-TA-S, GR-Lab and ES-Sus) were homozygous wildtype (SS) at the respective positions.

Strain (Genotype)	G4903 (SS) %	V4903 (SR) %	(RR) %	E4903 (SR) %	(RR) %	I4746 (SS) %	M4746 (SR) %	(RR) %	T4746 (SR) %	(RR) %	M/T4746 (RR) %
BCS-TA-S	100	0	0	0	0	100	0	0	0	0	0
GR-Lab	100	0	0	0	0	100	0	0	0	0	0
IT-GELA-SD4	0	0	33	0	67	4	16	80	0	0	0
GR-IER-15-2	70	0	20	0	10	76	24	0	0	0	0
ES-Sus	100	0	0	0	0	100	0	0	0	0	0
ES-MUR-14	100	0	0	0	0	67	33	0	0	0	0
BR-GML1	0	0	0	0	100[a]	67	0	0	0	33	0
BR-PSQ	0	0	0	0	10						

[a] Frequency estimated by Sanger sequencing (pooled n ≥ 4).

Strain ES-MUR-14 collected in Spain in 2014, which showed very low resistance to chlorantraniliprole (8-fold), was exclusively wild-type at position 4903 but carried a low frequency (33 %) of individuals heterozygous for I4746M.

3.4 Discussion

Diamide insecticides have been introduced for European tomato leafminer control only very recently and the first field failures were reported in greenhouse tomatoes in Italy in 2014 after repeated applications of chlorantraniliprole (Roditakis *et al.* 2015). The resistance cases in Italy are no longer restricted to greenhouse tomatoes in Sicily, but have now also been reported in other regions in the south, e.g. Puglia (Stefan Herrmann, Bayer CropScience, personal communication). Diamide resistance ratios of more than 1000-fold highlight the severity of the problem in Italy, triggered by repeatedly treating consecutive generations of this pest and failure to implement appropriate resistance management strategies as recently proposed (Teixeira and Andaloro 2013). *Tuta absoluta* was first reported in Europe (Spain) in 2006, but after only 5 years had spread to most tomato growing regions in the Mediterranean Basin, underlining its exceptional invasive potential (Desneux *et al.* 2011). Aggressive control measures comprising almost weekly insecticide applications targeting consecutive generations of the pest, known to complete 10–12 generations per year under greenhouse conditions, facilitated resistance development to diamides in a relatively short time (≤4 years), similar to observations made in repeatedly treated diamondback moth populations in cabbage fields in different geographic locations (Troczka *et al.* 2012, Wang and Wu 2012, Gong *et al.* 2014, Steinbach *et al.* 2015).

In this study we also investigated field-collected *T. absoluta* populations showing low to high levels of diamide resistance sampled outside of Italy, specifically Greece, Spain and Brazil. High levels of diamide resistance were also found in Brazilian populations, whereas moderate and low levels of resistance were detected in populations collected in Greece and Spain in 2015 and 2014, respectively. We went on to select one of the Italian strains collected in 2014 (IT-GELA-14-1) for additional generations with chlorantraniliprole and investigated the molecular basis of resistance in detail. Our study was informed by recent work conducted on diamide resistant diamondback moth populations, which revealed amino acid substitutions in the *Plutella* RyR transmembrane domain that confer target-site resistance (Troczka *et al.* 2012, Troczka *et al.* 2015, Guo *et al.* 2014, Gong *et al.* 2014, Steinbach *et al.* 2015). We isolated the full-length *T. absoluta RyR* gene and focused on the C-terminal domain spanning approx. 450 amino acids and containing six highly conserved transmembrane segments previously shown to play a major role in diamide insecticide binding (Kato *et al.* 2009). It was recently shown that the G4946E mutation in the diamondback moth RyR has strong functional implications for both the direct binding of tritiated diamide insecticides and their ability to allosterically enhance [³H]ryanodine binding in isolated thoracic microsomal membrane preparations (Steinbach *et al.* 2015). This was confirmed in a second study measuring calcium transients in diamide treated Sf9 insect cells stably expressing *Plutella* RyR constructs carrying

the G4946E mutation when compared to cells expressing wildtype receptors (Troczka *et al.* 2015).

Here we have discovered the corresponding mutation, G4903E, in the RyR of the diamide selected *T. absoluta* strain IT-GELA-SD4. Furthermore, we also identified an alternative amino acid substitution at the same position, G4903V, that has not been previously described. Both precisely correspond to the G4946E mutation site functionally proven to alter diamide binding in the RyRs of diamide resistance *Plutella*. As previously demonstrated for *P. xylostella* we confirmed the strong impact of mutations at this site using radioligand binding studies on thoracic membrane preparations of tomato leafminer adults of strain IT-GELA-SD4 in comparison to a susceptible reference strain (BCS-TA-S). This binding assay is considered to be a reliable and sensitive indicator of diamide action on insect RyRs (Lümmen *et al.* 2007 and Qi *et al.* 2014). Flubendiamide allosterically increased [^{3}H]ryanodine binding in RyR preparations of the susceptible strain BCS-TA-S at low nanomolar concentrations (EC_{50}-value: 3 nM), i.e. in the range of EC_{50}-values recently reported for other lepidopteran membrane preparations. The EC_{50}-value for [^{3}H]ryanodine binding stimulation by flubendiamide in strain IT-GELA-SD4 was at least 300-fold higher, thus confirming insensitivity and the relevance of the RyR G-to-E (or -V) target-site mutation in a species other than diamondback moth.

The IT-GELA-SD4 strain also carries an additional mutation, I4746M, at very high frequency. This corresponds to the I4790M mutation recently described in the RyR of diamide resistant populations of diamondback moth from China (Guo *et al.* 2014). In our study this mutation was present in combination with G4946E so the impact of I4790M on diamide binding and its contribution to resistance remains unclear. Nevertheless a recent study using homology modeling of the *Plutella* RyR (Steinbach *et al.* 2015), based on a recently published vertebrate RyR1 structure determined by single-particle electron cryomicroscopy (Yan *et al.* 2015), has suggested that both sites may contribute to diamide binding. The model suggests that the G4946E mutation is located at the interface between helix S4 and the S4–S5 linker, thought to have a critical role in RyR gating by impacting the movement of pore-associated helices (Ramachandran *et al.* 2013). The second mutation I4790M was shown to be located in helix S2 in close proximity to G4946E. Whereas G4946 is highly conserved in all insect species, methionine 4790 - described as a RyR mutation site in diamide resistant *P. xylostella* - is wildtype in the RyRs of several other insect species that are highly sensitive to chlorantraniliprole, a fact prompting Steinbach *et al.* (2015) to speculate that this site determines binding specificity and differences in selectivity profiles between anthranilic and phthalic diamides rather than field-relevant resistance (reviewed in Nauen and Steinbach 2016). Diamide resistance in *T. absoluta* strain IT-GELA-SD4 is autosomally inherited, and as an incompletely recessive trait (heterozygotes display a near susceptible phenotype), a finding corresponding to

the inheritance of diamide resistance mediated by target-site mutations in diamondback moth (Guo *et al.* 2014 and Steinbach *et al.* 2015).

To facilitate the genotyping of target-site resistance in *T. absoluta* field samples (e.g. conserved and shipped in alcohol) we developed a pyrosequencing method based on genomic DNA recently successfully used to monitor the geographic spread of RyR target-site resistance in diamondback moth (Steinbach *et al.* 2015). While pyrosequencing individuals for the presence of I4746M in both Brazilian strains included in this study, we detected another novel mutation, I4746T, carried in the homozygous form or as combined I4746 T/M heterozygotes. However, both Brazilian strains also expressed the G4903E RyR target-site mutation as revealed by Sanger sequencing of the respective domain in the *RyR* gene. Genotyping by pyrosequencing of the slightly resistant (8-fold) Spanish field strain ES-MUR-14 failed to detect G4903E (or 4903V) either in the heterozygous or homozygous form, whereas a Greek field strain collected in 2015 and exhibiting 55-fold resistance to chlorantraniliprole carried G4903V and G4903E in the homozygous form at low frequencies of 20 % and 10 %, respectively. Interestingly both these strains also carried the I4746M mutation at similar low frequency and in the heterozygous form. Given this finding it is likely that the greater level of diamide resistance in the Greek field strain GR-IER-15-2 is conferred by the low frequency of the G4903V and G4903E mutations in this strain. In the case of the Spanish strain we either did not pyrosequence enough individuals to detect rather low frequencies of G4903E/V genotypes, or another, less potent, mechanism of resistance might explain resistance. A possible candidate mechanism is enhanced detoxification by cytochrome P450s, as recently implicated in Brazilian *T. absoluta* strains exhibiting variation in monooxygenase activity correlated with their response to the diamides chlorantraniliprole and cyantraniliprole (Campos *et al.* 2015). However, most if not all studies on lepidopteran pests so far published, failed to clearly demonstrate strong evidence for metabolic mechanisms of diamide resistance powerful enough to cause field failure at recommended rates (Nauen and Steinbach 2016). This is largely supported by our results showing a lack of elevated levels of detoxification enzymes in the selected IT-GELA-SD4 strain when compared to a susceptible reference strain. However, in contrast to this finding, we observed notable synergism of chlorantraniliprole by DEF in strain IT-GELA-SD4 suggesting that esterases may play some role in enhancing chlorantraniliprole toxicity.

Tomato leafminer is the second lepidopteran pest species, after diamondback moth, to develop extremely high levels of diamide resistance mediated by confirmed RyR target-site mutations that confer sufficient levels of resistance to compromise efficacy under field conditions at recommended application rates. Diamides were only introduced to the market 10 years ago, but due to their high efficacy at low rates they quickly gained widespread adoption reflected in global sales and application frequency in certain agri- and horticultural settings (Teixeira and Andaloro 2013 and Sparks and Nauen 2015). Unfortunately, it is likely that additional crop pest

species are at high risk of developing target-site resistance to this insecticide class unless appropriate insecticide resistance management (IRM) strategies, particularly those based on mode of action rotation, are adhered to (Teixeira and Andaloro 2013, Sparks and Nauen 2015 and Nauen and Steinbach 2016). In this regard the implementation of IRM programmes to protect new modes of action such as RyR modulators from rapid resistance development needs to be an essential cornerstone of modern crop protection in order to prolong the life of the limited arsenal of actives currently available for control and guarantee sustainable crop yields.

See Appendix A for further data.

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Chapter 4

A resistance mutation conserved between insects and mites unravels the mode of action of benzoylurea insecticides inhibiting chitin biosynthesis

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Abstract

Despite the major role of chitin biosynthesis inhibitors such as benzoylureas (BPUs) in the control of pests in agricultural and public health for almost four decades, their molecular mode of action (MoA) has in most cases remained elusive. BPUs interfere with chitin biosynthesis and were thought to interact with sulfonylurea receptors that mediate chitin vesicle transport. Here, we uncover a mutation (I1042M) in the chitin synthase 1 (CHS1) gene of BPU-resistant Plutella xylostella at the same position as the I1017F mutation reported in spider mites that confers etoxazole resistance. Using a genome-editing CRISPR/Cas9 approach coupled with homology-directed repair (HDR) in Drosophila melanogaster, we introduced both substitutions (I1056M/F) in the corresponding fly CHS1 gene (kkv). Homozygous lines bearing either of these mutations were highly resistant to etoxazole and all tested BPUs, as well as buprofezin-an important hemipteran chitin biosynthesis inhibitor. This provides compelling evidence that BPUs, etoxazole, and buprofezin share in fact the same molecular MoA and directly interact with CHS. This finding has immediate effects on resistance management strategies of major agricultural pests but also on mosquito vectors of serious human diseases such as Dengue and Zika, as diflubenzuron, the standard BPU, is one of the few effective larvicides in use. The study elaborates on how genome editing can directly, rapidly, and convincingly elucidate the MoA of bioactive molecules, especially when target sites are complex and hard to reconstitute in vitro.

Significance

An old enigma in insect toxicology, the mode of action (MoA) of selective chitin biosynthesis inhibitors in arthropods, is resolved. Benzoylureas, buprofezin, and etoxazole share a MoA by directly interacting with chitin synthase 1. The finding that a single mutation confers striking levels of insecticide resistance against three putative different MoAs has important ramifications on resistance management strategies and rational use of insecticides against major agricultural pest*s* and vectors of human diseases. Our results also show that CRISPR/Cas9-mediated gain-of-function mutations in single-copy genes of highly conserved target sites in arthropods provide opportunities for comprehensive insecticide resistance investigations across species boundaries and against several insecticide classes.

4.1 Introduction

Insects pose tremendous threats to humans in two main areas. Pathogens causing diseases such as malaria, dengue fever, and more recent problems caused by the Zika virus, are vectored by mosquitos, such as the *Anopheles gambiae* and *Aedes aegypti*, and cause severe global health problems (WHO 2016). Furthermore, the sustainability of agricultural yields, which need to meet predicted population growth (FAO 2010), is seriously threatened by pest insects and mites. The diamondback moth *Plutella xylostella*, a global lepidopterous pest of brassicaceous vegetables, is one of the economically most important agricultural pests in the world, particularly due to it having developed resistance to almost all chemical classes of insecticides applied for its control under continuous insecticide pressure (Talekar and Schelton 1993).

Protection of food sources and human health from invertebrate pests is critically reliant on insecticides (Sparks and Nauen 2015, Bhatt *et al.* 2015). Insecticides are classified according to mode of action (MoA) and chemistry into several groups through the IRAC (Insecticide Resistance Action Committee) insecticide grouping system, which is the basis for their rational use and resistance management strategies (Sparks and Nauen 2015). The vast majority of current insecticides have neurotoxic and muscle action (>80 %), whereas only a relatively small proportion interfere with growth and development (insect growth regulators, IGRs) and thus are highly selective to targeted arthropod pests as there are often no physiologically related processes or target sites present in vertebrates. IGRs are a group of chemically diverse compounds including the microbial-derived pyrimidine-nucleoside peptides, benzoylureas (BPUs), oxazolines, and thiadiazines (Merzendorfer 2013) that all interfere with chitin biosynthesis or transport and deposition pathways. The MoA of the antifungal pyrimidine-nucleoside antibiotics is by their function as substrate analogs of UDP-*N*-acetylglucosamine at the catalytic site of chitin synthase (CHS) and are thus considered competitive inhibitors (Cohen and Casida 1980, Zhang and Miller 1999, Ruiz-Herrera and San-Blas 2003). BPUs (Sun *et al.* 2015), such as the major mosquito larvicide diflubenzuron and the agriculturally widely used insecticides triflumuron and lufenuron, represent a group of compounds (group 15 with regard to the IRAC grouping system; see also Appendix B: Fig. S1) that inhibit chitin biosynthesis by a unique yet elusive mechanism of action independent of the catalytic reaction of CHS itself (Cohen 2010, Merzendorfer 2013, Sun *et al.* 2015). Although the sulfonylurea receptor (SUR) has been suggested as the direct target of BPUs (Matsumura 2010) by affecting chitin biosynthesis indirectly by altering vesicle trafficking, its role in chitin biosynthesis inhibition remains controversial (Akasaka *et al.* 2006, Gangishetti *et al.* 2009). Furthermore, it was recently shown that SUR is dispensable for cuticle formation and chitin biosynthesis in *Drosophila melanogaster* (Meyer *et al.* 2013).

Buprofezin (group 16) and etoxazole (group 10B) are two other, chemically different compounds (Appendix B: Fig. S1) highly selective to sucking agricultural pests that have also

been proposed to interfere with chitin biosynthesis or cuticle formation (Uchida *et al.* 1985, De Cock and Degheele 1991). Etoxazole is an oxazoline acaricide widely used against pest mite species but with limited activity on insects (Nauen and Smagghe 2006). Genomic mapping of a recessive monogenic etoxazole resistance locus in the two-spotted spider mite *Tetranychus urticae*, together with additional genetic and biochemical evidence, suggests that a single mutation in *CHS1* is associated with etoxazole resistance; this mutation, I1017F (*T. urticae* numbering), is located in the C-terminal transmembrane domain. Therefore, it is likely that CHS1 is the molecular target of etoxazole as well as the chemically different acaricides clofentezine and hexythiazox (Van Leeuwen *et al.* 2012, Demaeght *et al.* 2014). Based on the similarity of symptoms for poisoning observed following exposure to both BPUs and etoxazole, as well as their inhibitory potential on chitin biosynthesis in isolated integuments of lepidopteran larvae, it has been hypothesized that they share the same MoA (Nauen and Smagghe 2006). The same direct MoA of BPU on CHS1, but not SUR, was later also postulated (Van Leeuwen *et al.* 2012). However, no molecular evidence for such a possible association exists; there have been reports of BPU resistance in the diamondback moth in subtropical areas with intensive use of BPUs (Teixeira and Andaloro 2013), but the molecular mechanism remains unknown. Furthermore, functional evidence of the involvement of the I1017F mutation in resistance could not be provided, given that in vitro approaches using recombinant protein expression are not feasible for large oligomeric integral protein complexes, especially when interactions are pre- or postcatalytic or involve the oligomerization of the complex (Merzendorfer 2013, Van Leeuwen *et al.* 2012). As functional evidence is missing, the MoA through which chitin biosynthesis inhibitors exert their insecticidal activity remains uncertain.

Recent advances in genome modification technology, and especially the emergence of CRISPR/Cas9 (Jinek *et al.* 2012), allow the application of "reverse" genetics approaches to provide in vivo evidence of the linkage between genotypes with phenotypes, including the study of insecticide MoA via generation of gain-of-function/loss-of-function mutations.

Here, we study and further select BPU resistance in *P. xylostella* and analyze the genetics of resistance as well as the possible association of identified point mutations in its *CHS1* gene with the phenotype. We use CRISPR to generate the corresponding single mutations associated with BPU (and etoxazole) resistance in *D. melanogaster*, a model organism that is equipped with an efficient genetic "toolbox" enabling the fast and reliable study of the contribution of individual mutations to resistance. Toxicity bioassays with genome-modified flies are used to reveal insensitivity to BPUs and buprofezin, thus attempting to provide compelling evidence for the functional interaction with CHS1 as the molecular target site.

4.2 Material and methods

4.2.1 Chemicals

Insecticides (diflubenzuron, triflumuron, lufenuron, and flucycloxuron) used for *P. xylostella* bioassays were of technical grade (purity >98%) and provided in-house (Bayer CropScience). Commercial insecticide formulations were used for *Drosophila* bioassays, namely Borneo [11% (wt/vol) etoxazole; Hellapharm], Dimilin [48% (wt/vol) diflubenzuron; Syngenta], Match [50% (wt/vol) lufenuron; Syngenta], Trigard [75% (wt/vol) cyromazine; Syngenta], and Applaud [25% (wt/vol) buprofezin; Syngenta]. All other chemicals were purchased from Sigma-Aldrich.

4.2.2 Insects

The susceptible reference strain (BCS-S) of *P. xylostella* L. (Lepidoptera: Plutellidae) has been maintained under laboratory conditions for more than 20 years without exposure to insecticides. Strain Sudlon was collected in a cabbage field located in Sudlon, Cebu Island, in the Philippines in 2011 as described elsewhere (Steinbach *et al.* 2015). The BPU-resistant strain Sudlon-Tfm was obtained by selecting strain Sudlon for 10 generations with triflumuron by incrementally increasing its concentration to 1,000 mg·L^{-1}. The Japan strain was collected in Mizobe, Japan in 2010. Finally, the strains from China and India were collected from cabbage in 2014. All strains were maintained on cabbage plants (*Brassica oleracea*) as recently described (Steinbach *et al.* 2015). Strain Sudlon-Tfm was maintained on triflumuron- (1,000 mg·L^{-1}) treated cabbage plants. The *Drosophila* strain y^1 M{nos-Cas9.P}ZH-2A w* (nos.Cas9; stock no. 54591 at Bloomington Stock Center) (Port *et al.* 2014) as well as yw strain and the strain yw; TM3 *Sb e*/TM6B *Tb Hu e* (containing third chromosome balancers, provided by Christos Delidakis, Institute of Molecular Biology and Biotechnology/ Foundation for Research and Technology Hellas and University of Crete, Heraklion, Crete, Greece) were used in this study. *Drosophila* strains were typically cultured at 25 °C temperature, 60–70 % humidity, and 12/12-h photoperiod on standard fly diet.

4.2.3 Bioassays

Leaf dip bioassays with third instar diamondback moth larvae were conducted after IRAC method no. 7 (www.irac-online.org) as described recently (Steinbach *et al.* 2015). Control mortality was less than 10 %. LC$_{50}$ values and their corresponding 95% fiducial limits were calculated using Prism 5.03 (GraphPad Software, Inc.). For *Drosophila* bioassays, second instar larvae were collected and transferred in batches of 20 into new vials containing fly food supplemented with different insecticide concentrations. Larval development, molting, pupal eclosion, and adult survival were monitored for a period of 10–12 d. Five to six insecticide concentrations that cause 5–95 % mortality (when applicable) were tested in triplicate, together

with relevant negative (no insecticide) controls, in genome-modified flies and wild-type (nos.Cas9 and/or yw) controls. Dose-dependent molting and/or mortality curves were constructed from dose–response data, and LC_{50} values were calculated with PoloPlus (LeOra Software). A χ^2 test was used to assess how well the individual LC_{50} values agreed with the calculated linear regression lines.

4.2.4 Crossing Experiments

Pupae of strains BCS-S, Sudlon, and Sudlon-Tfm were collected and kept in Petri dishes individually until they hatched. After sex determination, 50 virgin females of Sudlon-Tfm were crossed with 50 males of Sudlon strain or BCS-S strain and vice versa. Because there was no difference obtained between the two reciprocal crosses, the F1 generation was pooled for further studies. The F1 generation was backcrossed with the respective parental strains. The backcross was conducted following the same approach as the reciprocal crosses; there was no difference obtained among the offspring, so samples were pooled. Third instar larvae were used for leaf dip bioassays to obtain the individual LC_{50} values for triflumuron. The degree of dominance (D) was calculated using Stone's equation. (Stone 1968). Larvae of the different strains were preserved in RNA*later* (Ambion) and analyzed for the I1042M/F mutation by pyrosequencing.

4.2.5 Pyrosequencing

Individual *P. xylostella* larvae were ground in lysis buffer, and total genomic DNA (approximately 400 ng per larvae) was extracted using DNAdvance Tissue Kit (Agencourt) according to the to the supplier's recommended protocol. A gene fragment of 210 bp was amplified by PCR from 50-ng aliquots of gDNA using the primer pair PxCHS1-forward and PxCHS1-reverse (Appendix B: Table S2), designed with Assay Design Software (PSQ-Biotage AB, now Qiagen). The primer pair is based on a ClustalW aligned consensus sequence of *CHS1* of diamondback moth found in GenBank (accession number AB271784) as well as internally sequenced *CHS1* of strains BCS-S, Japan, and Sudlon. The pyrosequencing protocol comprised 35 PCR cycles with 0.5 μM forward and biotinylated reverse primer in 30 μL reaction mixtures containing 1× Taq enzyme reaction mix (RedTaq Jumpstart Master Mix, Sigma-Aldrich) and cycling conditions of 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final elongation step at 72 °C for 5 min. The single-strand DNA required for pyrosequencing was prepared as described in Troczka *et al.* (2012). The pyrosequencing reactions were carried out according to the manufacturer's instructions using the PSQ 96 Gold Reagent Kit (Qiagen), and the sequence-PxCHS1-seq (Table S2) for genotyping. The pyrograms were analyzed using the SNP Software (Qiagen).

4.2.6 Genomic Engineering Strategy

An ad hoc CRISPR/Cas9 genomic engineering strategy was devised to generate the I1056M/F mutations (equivalent to the I1042M and I1017F mutation in *P. xylostella* and *T. urticae CHS1*, respectively; Fig. 4.2, Appendix B: *SI Materials and Methods* and Fig. S5) at the *kkv* gene in *D. melanogaster*. Potential CRISPR targets in the region of interest were identified using the online tool Optimal Target Finder (Gratz *et al.* 2014) (tools.flycrispr.molbio.wisc.edu/targetFinder/), and two targets with no predicted off-target hits were selected to generate RNA expressing plasmids gRNA444 and gRNA658, respectively, targeting the relevant genomic regions (Appendix B: *SI Materials and Methods* and Fig. S5). We constructed de novo (Genscript) two donor plasmids for HDR, encompassing genomic region 3R:5380538:5383542 but with certain modifications compared with the wild-type genomic sequence (Appendix B: Fig. S5).

4.2.7 *Drosophila* DNA Purification and Amplification.

DNA was purified from *Drosophila* tissues by DNAzol (MRC) according to the manufacturer's instructions. PCR amplification with relevant primer pairs (Appendix B: Table S2) was typically performed with Kapa Taq DNA Polymerase (Kapa Biosystems). The conditions used were 95 °C for 2 min, followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min followed by a final extension step for 2 min.

4.2.8 Generation and Selection of Genome-Modified Flies.

We used transgenic flies with the genotype y^1 M{nos-Cas9.P}ZH-2A w* that carry a transgene expressing Cas9 protein during oogenesis under control of *nanos* regulatory sequences (Port 2014) and injected embryos as described in *SI Materials and Methods*. Screening was performed by isolating DNA from sets of ~30 individuals per vial (mostly pupae, but also adults and third instar larvae, depending on availability). In case the presence of genome-modified alleles was indicated in the pool (Appendix B: Fig. S6), several individual G_1 flies from the same original cross were first crossed again with nos.Cas9 flies to generate G_2 progeny and then individually screened to positively identify which of these G_1 flies indeed carried genome-modified alleles. Lines originating from positive G_1 flies were established, and individual G_2 flies (expected to be heterozygous for the mutant allele at a 50 % ratio) were balanced against a strain containing third chromosome balancers (yw; TM3 *Sb e*/TM6B *Tb Hu e*). Flies potentially containing modified alleles were screened as before after being back-crossed to the original balancer stock (Appendix B: Fig. S6); the progeny of positives (bearing the modified allele opposite to one of the balancer chromosomes) was used to generate

homozygous lines by crossing between siblings and selecting against the marker phenotype (*Sb* or *Tb Hu*) for the relevant balancer. All lines used were sequence-verified.

4.3 Results

4.3.1 Selection and Characterization of BPU Resistance in *P. xylostella*.

Low but significant resistance levels against diflubenzuron and triflumuron were detected in a *P. xylostella* strain (Sudlon) recently sampled in a Philippine cabbage field. The strain was maintained under laboratory conditions since 2011 to investigate target-site mutations in ryanodine receptors conferring resistance to diamide insecticides (Troczka *et al.* 2012, Steinbach *et al.* 2015). BPU insecticides have been used for diamondback moth control in Philippine cabbage in the past and were recently abandoned due to development of resistance. The Sudlon strain was reselected with triflumuron under laboratory conditions, resulting in the strain Sudlon-Tfm. Selection for 10 generations resulted in high BPU cross-resistance compared with the parental strain and reference strains BCS-S and Japan (Table 4.1). The selected strain Sudlon-Tfm was not only resistant to chemically diverse BPUs but also etoxazole (>178-fold), a chitin biosynthesis inhibitor of a different chemical class. Reciprocal crosses between Sudlon-Tmf and BCS and Sudlon revealed that the resistance was inherited autosomal recessive (Fig. 4.1) with a degree of dominance ranging from –0.73 to –0.88 in all reciprocal crosses (Appendix B: Table S1). Comparison of the postembryonic developmental time of strains Sudlon and Sudlon-Tfm showed that Sudlon-Tfm had a significantly longer larval (fourth instar) and pupal development time (Appendix B: Fig. S2), which could be indicative of possible fitness costs associated with the selected BPU resistance trait in *Plutella*.

Based on (*i*) the identical symptoms of poisoning observed following exposure to both BPUs and etoxazole, (*ii*) the inheritance of resistance in an autosomal and recessive way in line to the etoxazole resistance phenotype previously reported in spider mites (Van Leeuwen *et al.* 2012), and (*iii*) the strong genetically based evidence that etoxazole likely acts on CHS1 but not SUR (Van Leeuwen *et al.* 2012), we subsequently cloned and sequenced the full-length *CHS1* gene of *P. xylostella* strains BCS-S (GenBank accession no. KX420688), Sudlon (GenBank accession no. KX420689), and Sudlon-Tfm (GenBank accession no. KX420690) to compare the sequences between BPU-resistant and -susceptible strains.
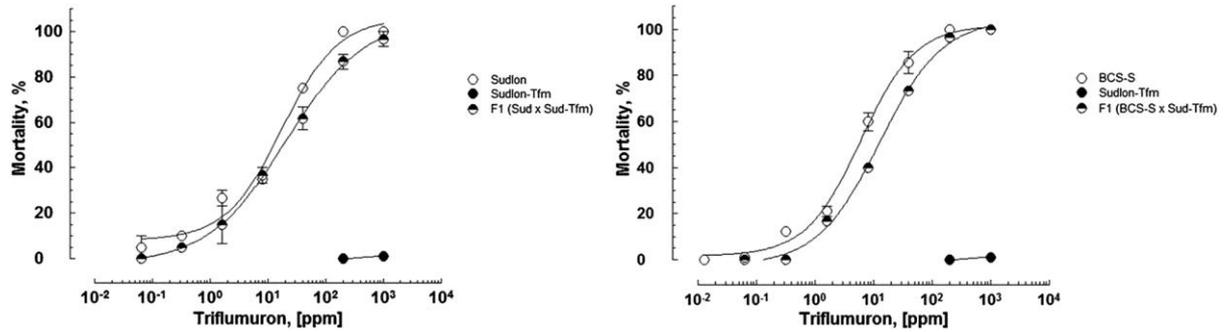
Figure 4.1. Log-dose mortality data for triflumuron tested against third instar larvae of diamondback moth strains BCS-S, Sudlon, and Sudlon-Tfm as well as combined reciprocal crosses (F1). Error bars represent SEM.

Table 4.1. Log-dose probit mortality for commercial BPU insecticides and etoxazole tested against third instar larvae of different strains of diamondback moth in leaf-dip bioassays (96 h).

Compound	Strain	N	LC$_{50}$, ppm	95% CL*	Slope	RR†
Diflubenzuron	BCS-S	300	36	21.0–60.3	1.3	
	Japan	300	45	24–85	1.2	1
	Sudlon	300	317	118–855	1.2	9
	Sudlon-Tfm	300	>1,000			>28
Triflumuron	BCS-S	420	5.3	4.2–6.9	1	
	Japan	420	11.6	7.8–17.3	0.89	2
	Sudlon	420	17.6	10.5–29.5	0.88	3
	Sudlon-Tfm	180	>1,000			>188
Lufenuron	BCS-S	450	1.8	0.96–3.5	1.3	
	Japan	420	1.2	0.28–4.7	0.47	1
	Sudlon	390	0.63	0.2591–1.510	0.86	1
	Sudlon-Tfm	330	354	57–2189	0.94	196
Flucycloxuron	BCS-S	240	0.16	0.15–0.18	1.3	
	Japan	240	0.36	0.21–0.63	1.6	2
	Sudlon	240	0.091	0.068–0.12	1.1	1
	Sudlon-Tfm	540	179	27–1183	0.50	1,119
Etoxazole	BCS-S	120	2.8	1.7–4.4	0.99	
	Sudlon	120	5.3	3.2–8.7	0.99	2
	Sudlon-Tfm	120	>500			>178

* 95% confidence limits.

† Resistance ratio (based on strain BCS-S).

Compared with the *CHS1*, cDNA sequence of both susceptible strains BCS-S and Sudlon, a single nonsynonymous SNP resulting in a isoleucine (I)-to-methionine (M) amino acid change at position 1042 (*P. xylostella* numbering) in the C-terminal region of CHS1 of strain Sudlon-Tfm was found (Fig. 4.2). Genotyping of individual larvae by pyrosequencing of amplified *CHS1* fragments covering that region revealed that the I-to-M amino acid substitution at position 1042 (I1042M), which was completely absent in the BCS-S strain, was present at low

frequency in the Sudlon strain and fixed (100 %) in the resistant Sudlon-Tfm strain after selection with triflumuron (Table 4.2 and Appendix B: Fig. S3).
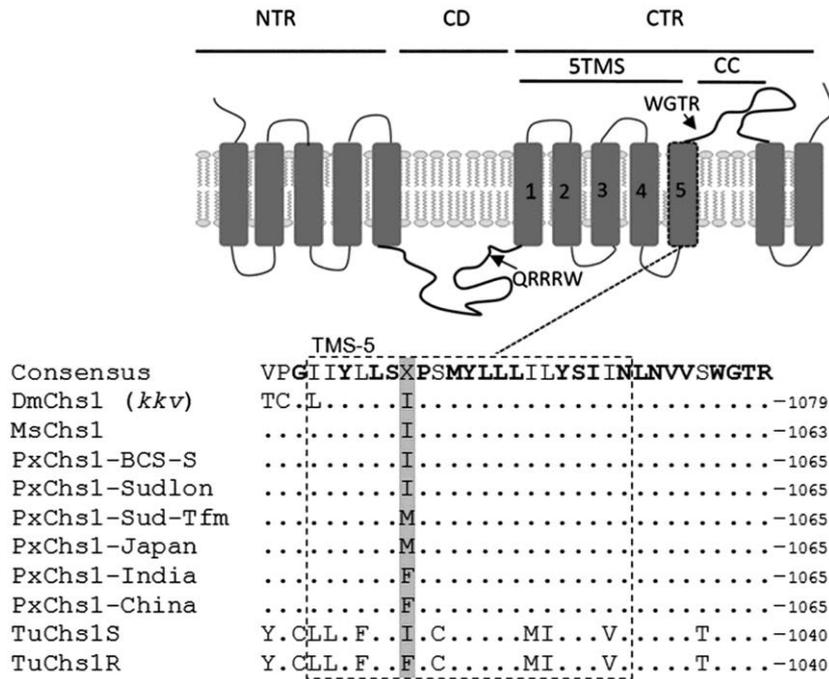
Figure 4.2. Location of the two mutations conferring resistance. (*Top*) Schematic representation of domain architecture of CHS1, redrafted from Van Leeuwen *et al.* 2012. 5TMS, cluster of five transmembrane segments; CC, coiled-coil motif; CD, catalytic domain; CTR, C-terminal region; NTR, N-terminal region. Rectangular boxes represent transmembrane domains. Arrows point to signature sequences QRRRW (catalytic domain) and WGTR (N-terminal region). (*Bottom*) Aligned amino acid sequences of helix 5 in the 5TMS clusters of CHS1 of *D. melanogaster* (Dm), *M. sexta* (Ms), six strains of *P. xylostella* (Px), and *T. urticae* (Tu; S, etoxazole susceptible; R, etoxazole resistant). Conserved residues are shown in bold. The position of the I1042M/F substitution in resistant *P. xylostella* (I1017F in etoxazole-resistant mites) is indicated in gray.

The frequency of the 1042M/1042M alleles was 7 % in a population from Japan, whereas the frequency of the 1042M/1042M in survivors of BPU treatment (>100 ppm) of the same population was 100 % (Table 4.2). The correlation between mutation and resistance is significant ($R^2 = 0.9779$, $P = 0.0002$). The I1042M mutation was also present at relatively high frequencies in field populations of *P. xylostella* sampled from cabbage fields in China and India with known BPU control failures (Table 4.2). Furthermore, genotyping of amplified *CHS1* fragments of individual larvae of the Chinese field strain revealed another mutation, I1042F, which has been associated (Van Leeuwen *et al.* 2012) with etoxazole resistance in *T. urticae* (Fig. 4.2, corresponding position I1017F).

Table 4.2. Genotyping (individual larvae) by pyrosequencing for a CHS1 target-site mutation (I1042M) in different strains of diamondback moth.

Strain	N	SS I1042, ATT/ATT	RS I/M1042, ATT/ATG	RR M1042, ATG/ATG	RR L1042, TTG	RR M/L1042, ATG/TTG	RR M/F1042, ATG/TTT
					Frequency of genotype, %		
BCS-S	30	100	0	0			
Sudlon	30	97	0	3			
Sudlon-Tfm	40	0	0	100			
Japan	30	50	43	7			
Japan[*]	6	0	0	100			
Px-China	59	27	20	25	3	2	22
Px-India	23	52	30	17			
Reciprocal crosses							
F1-A (BCS-S × Sudlon-Tfm)	40	0	100	0			
F1-B (Sudlon × Sudlon-Tfm)	63	0	89	11			

[*] Survivors of BPU treatment (>100 ppm).

4.3.2 *Drosophila* Flies Bearing the Mutations Corresponding to I1042M and I1017F Are Resistant to BPUs and Other Chitin Biosynthesis Inhibitors.

We identified the ortholog CHS gene in *Drosophila* (*krotzkopf verkehrt* or *kkv*; Appendix B: *SI Results* and Fig. S4), and to generate in *kkv* the I1056F/M mutations corresponding to I1017F in *T. urticae* and I1042M in *P. xylostella*, respectively (Fig. 4.2), we injected strain y[1] M{nos-Cas9.P}ZH-2A w* (referred to as nos.Cas9 below) embryos with the appropriate gRNAs/donor plasmid mixes (Appendix B: *SI Materials and Methods* and Fig. S5) and screened progeny for genome-modified alleles. For the I1056F mutation, there were indications for the presence of homology-directed repair (HDR)-derived alleles within the sample at 16 different lines—that is, ∼20 % of the total number (i.e., 77) of lines that gave G_1 progeny. G_1 individuals from each of three different original (G_0) lines were crossed to balancer flies and screened to identify positive heterozygotes (Appendix B: Fig. S6). Several independent lines were established, and at least one became readily homozygous after balancing (line Et15); this line was verified by sequencing the relevant genomic region and shown to be genome-modified as expected, carrying the I1056F mutation at the *kkv* gene. Similarly, for the I1056M mutation, HDR-derived alleles were found (Appendix B: Fig. S6) in pools from 16 lines out of the 48 screened (∼33 %), and individuals from three lines were crossed to balancers and screened. Several lines were sequence-verified as homozygous; line Px39 was selected for conducting toxicity bioassays.

Toxicity assays with *Drosophila* larvae of strains nos.Cas9 and *yw* (both of which contribute to the genetic background of genome-modified flies) indicated that the strains carrying the wild-type *kkv* allele were sensitive to etoxazole at concentrations around 10 mg/L, without any significant differences observed between the two strains. Larvae did not manage to pupate or even grow to third instar. On the contrary, larvae from the genome-modified strains Et15 and Px39 bearing either the I1056F or I1056M homozygous mutation managed to grow and undergo molting without any visible problem, virtually all pupated, and adults eclosed normally when exposed to etoxazole concentrations as high as 10,000 mg/L, although at the highest concentrations (>1,000 mg/L) adults were dying just after eclosion. The LC_{50} values (with their corresponding 95% fiducial limits) for the susceptible (nos.Cas9) and resistant (Et15, Px39) lines and the associated resistance ratios are shown in Table 4 3.

Bioassay screens indicated a gross difference in the toxicity between both Px39 (I1056M) and Et15 (I1056F) *Drosophila* lines for diflubenzuron (LC_{50} nos.Cas9, 0.322 mg/L vs. LC_{50} Et15 and LC_{50} Px39, >5,000 mg/L), lufenuron (LC_{50} nos.Cas9, 0.148 mg/L vs. LC_{50} Et15, 16.659 mg/L and LC_{50} Px39, >20 mg/L), and buprofezin (LC_{50} nos.Cas9, 53.2 mg/L vs. LC_{50} Et15, >1,000mg/L and LC_{50} Px39, 1,276.654 mg/L). Such levels of at least partial cross-resistance support a common MoA between etoxazole, BPUs, and buprofezin. However, cyromazine toxicity is not affected either by the I1056M or the I1056F mutation, indicating either a different binding mode or another MoA.

The genome-modified fly lines used for bioassays were examined for certain life table parameters (Appendix B: *SI Materials and Methods* and Fig. S7), but no significant difference was observed in the flies bearing the I1042M mutation, contrary to the result from *Plutella* (Appendix B: Fig. S2).

Table 4.3. Bioassay results (LC$_{50}$ values and associated resistance ratios) of genome-modified flies (Et15, Px39) versus relevant unmodified controls (nos.Cas9) for five different insecticides.

Insecticides	Strains	LC$_{50}$, ppm (95% CL)	Resistance ratio
Etoxazole	Et15 (I1017F)	>10,000	>1,077
	Px39 (I1042M)	>10,000	>1,077
	nos.Cas9	9.28 (0.73–14.00)	1
Diflubenzuron	Et15 (I1017F)	>5,000	>15,625
	Px39 (I1042M)	>5,000	>15,625
	nos.Cas9	0.32 (0.24–0.42)	1
Lufenuron	Et15 (I1017F)	16.66 (8.70–66.47)	111.06
	Px39 (I1042M)	>20	>133
	nos.Cas9	0.15 (0.11–0.18)	1
Cyromazine	Et15 (I1017F)	0.23 (0.21–0.25)	0.74
	Px39 (I1042M)	0.30 (0.21–0.41)	1
	nos.Cas9	0.31 (0.25–0.34)	1
Buprofezin	Et15 (I1017F)	>1,000	>18.79
	Px39 (I1042M)	1,276.65 (1,110.36–1,554.15)	24.07
	nos.Cas9	53.20 (41.24–65.72)	1

4.4 Discussion

Resistance against the major chitin biosynthesis inhibitor class of insecticide chemistry (i.e., BPUs) was detected and subsequently selected in a recently collected Philippine field population of the diamondback moth *P. xylostella*, one of the most important agricultural pests in brassicaceous crops worldwide. The presence and frequency of the amino acid substitution I1042M was highly correlated with cross-resistance against several BPUs such as diflubenzuron, triflumuron, lufenuron, and flucycloxuron. Surprisingly, the *P. xylostella* mutation in *CHS1* gene lies at the same location of a previously documented mutation (I1017F) conferring etoxazole resistance (Van Leeuwen *et al.* 2012). Introduction of either mutation in *D. melanogaster* by a CRISPR/Cas9 coupled with HDR genome modification approach showed a similar resistance phenotype across different chemical classes of IGRs, such as BPUs, etoxazole, and buprofezin, but not cyromazine. This is compelling evidence that BPUs, buprofezin, and etoxazole share the same MoA and directly interact with CHS1.

Our chosen genetic validation approach is further supported by a contemporary study showing that the introduction of a single point mutation in an *alpha*6 subunit of the nicotinic acetylcholine receptor of *Drosophila* by CRISPR/Cas9 genome editing copying a mutation associated with spinosad resistance in thrips resulted in a spinosad-resistant phenotype in genome-modified flies (Zimmer *et al.* 2016). Our results show that a reverse genetics strategy is exceptionally suitable for the elucidation of the MoA of insecticides and/or functional validation of mutations associated with insecticide resistance in a wide array of targets that are otherwise difficult to study. CRISPR/Cas9 has already been used in *Drosophila* for resistance research

before (Zimmer *et al.* 2016, Somers *et al.* 2015). However, in this study, we have generated lines bearing homozygous recessive gain-of-function mutations in a single-copy gene, thus enabling comprehensive investigation—that is, comparative bioassays for these particular mutations against several insecticide classes. The fact that most target sites between arthropods are highly conserved allows screening of different mutations across species boundaries. This strategy has several potential valuable ramifications, as it can be used in a large number of molecular targets and a wide array of chemical classes of insecticides.

Procedures toward the investigation of insecticide MoA and resistance mechanisms typically involve in vitro screening systems (Troczka *et al.* 2015), electrophysiology (Dong *et al.* 2014), direct ligand/receptor–insecticide interactions either in vivo (Steinbach *et al.* 2015) or in silico (O'Reilly *et al.* 2006), functional expression of enzymes (Nauen *et al.* 2015, Riga *et al.* 2015), or genetic mapping linkage analysis (Van Leeuwen *et al.* 2012, Heckel 2003). However, there are cases where in vitro screening is not applicable because the native proteins or protein complexes cannot be reconstituted or recombinantly expressed. One such example is CHS1 because of its structure as a large oligomeric integral membrane protein that catalyzes both polymerization of sugars and translocation of the nascent chitin fiber across the plasma membrane. No structural information is available on CHS1 complexes, and even the quaternary structure is not known (although trimeric complexes have been purified from *Manduca sexta*, they could be building blocks of higher order complexes) (Maue *et al.* 2009), thus rendering impossible any effort to model interactions. Attempts in recombinant expression have failed to generate active complexes. In this and other cases, the interaction between target site and insecticides can be more complex than simply inhibiting natural substrate or ligand binding, making it even harder to develop a functional screening assay.

The elucidation of the MoA of the chitin biosynthesis inhibitor classes BPU and buprofezin (i.e., IGR insecticides) that have been used against major agricultural pests and disease vectors for many years, directly acting on CHS, as well as the identification of BPU target-site resistance mutations has important implications and impact for the rational use of insecticides and insecticide resistance management. It will directly affect the IRAC classification (Sparks and Nauen 2015) of those molecules, which are currently assigned to a MoA group (MoA group 15) different from etoxazole (MoA group 10) and buprofezin (MoA 16). Our study provides compelling evidence that both classes affect the same target protein, CHS1, thus justifying their subgrouping in a single MoA class. The finding that a single mutation confers high levels of insecticide resistance against three putative different MoAs has important effects on resistance management strategies, which are largely based on rotation of insecticide MoA groups, to avoid selection for target-site resistance by repeatedly applying chemistries addressing the same binding site.

The presence of the *CHS1* resistance mutation in diamondback moth populations from different countries, in particular, is an important consideration for rational use and management of insecticides against this major pest. The slightly but significantly extended development time of fourth instar larvae and pupae in strain Sudlon-Tfm indicated a putative fitness cost in *Plutella*, possibly associated with this mutation. However, this was not confirmed in *Drosophila* lines, where the mutation was isolated in an isogenic background. It is possible that unrelated genetic loci in the multiresistant Sudlon-Tfm laboratory strain (Steinbach *et al.* 2015) might have contributed to the fitness cost observed.

The developed pyrosequencing diagnostic as well as possible additional field-applicable technologies to detect the presence of *CHS1* target-site mutations provides a tool allowing us to screen rapidly for the presence of resistant genotypes to adjust resistance management strategies based on MoA rotation accordingly.

The findings may also have implications for public health insecticide-based vector control interventions. The larvicide diflubenzuron is one of the most important insecticides that have been used against mosquitoes, particularly in regions such as Europe, where neurotoxic insecticides are banned from use in mosquito breeding sites. Screening of *A. aegypti* and *Aedes albopictus* populations, the major vectors of arbovirus including Dengue and Zika, from several geographical regions for possible resistant *CHS1* alleles will guide appropriate resistance management strategies to ensure the sustainability of control interventions. This discovery will also potentially have a bearing on the choice of insecticide for new human pathogen vector control, such as against the malaria mosquito *A. gambiae s.s* (Hemingway *et al.* 2016).

For further information see Appendix B.

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Chapter 5

Fitness costs and life table parameters of highly insecticide resistant strains of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) at different temperatures

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Abstract

In many cases resistance alleles have been associated with fitness costs and are often dependent on environmental factors such as temperature. Here, we studied the effects of temperature on the overall fitness, including development, survival, and reproduction of three insecticide resistant and one susceptible strain of diamondback moth (DBM), Plutella xylostella (L.).

The broader cross-resistance profile of the resistant strains previously selected by diamide and benzoylurea insecticides was tested. Cohort studies were conducted in the laboratory at three different temperatures: 20 °C ± 1 °C, 25 °C ± 1 °C and 30 °C ± 1 °C and involved fitness costs were estimated. We observed significant differences in the development time where the susceptible strain had shown a shorter developmental period from egg stage to adult stage compared to the resistant strains. Moreover, the resistant strains differed significantly between one another. Additionally, the population growth parameters varied among the strains where the benzoylurea resistant strain had shown the highest costs affecting the overall fitness of this strain. 30 °C was unfavourable for DBM development resulting in a reduced fitness in all strains.

Benzoylurea selection pressure on a diamide-resistant P. xylostella strain resulted in lowest reproduction parameters and the longest generation time as well as doubling time among all strains tested. Thus, suggesting significant effects on the overall fitness and population growth parameters for diamide resistant populations pressured by benzoylureas under applied conditions.

5.1 Introduction

Diamondback moth*, Plutella xylostella* (L.) is a major lepidopteran pest of important cultivated brassicas worldwide. It is extremely destructive and if not controlled by chemical measures, crop losses can exceed 90 % (Talekar and Shelton 1993, Verkerk and Wright 1996). Yield losses of summer cabbage were 99 % and 80 % in 1992 and 1994, respectively in Shanghai (China) when no insecticides were applied to control DBM (Zhao *et al.* 1996). In the early 1990's more than a billion US Dollars were invested in insecticide control measures against DBM (Talekar and Shelton 1993). The extensive use of insecticides results in a high selection pressure on insects which in turn facilitates the spread of alleles that confer an adaptation to this environmental stress factor. So far DBM has been reported to be resistant against 95 different insecticides of more than ten mode of action classes (www.pesticideresistance.org, December 2016) with rising tendency which has major implications for integrated pest management. However, most insecticide resistance mechanisms are associated with fitness costs as these adaptive changes often have measurable effects on the overall fitness of a resistant insect compared to a susceptible counterpart. The fitness costs resulting from insecticide resistance are more or less dependent on environmental factors which have a great impact on life history traits, especially when the environment is unfavourable for insect development the estimated costs tend to be high. Such environmental stressors, like high or low temperatures (Li *et al.* 2007, Zhang *et al.* 2015), parasitism (Raymond *et al.* 2007) and food quality (Janmaat and Myers 2005, Golizadeh *et al.* 2009, Farahni *et al.* 2011) were shown to negatively affect developmental time, fertility and mortality and thereby limiting population growth. Moreover, negative genetic trade-offs can also be apparent in the absence of the insecticide or in the presence of sublethal doses (Hoffmann and Parsons 1991, Ribeiro *et al.* 2014). In *P. xylostella*, fitness costs have been shown to be a direct consequence of diamide resistance (Han *et al.* 2012, Yan *et al.* 2014) which, for example, resulted in a decline in the overall fitness in a cyantraniliprole-selected laboratory strain with negative effects on developmental time of larvae, rate of pupation, adult emergence and fertility (Liu *et al.* 2015). On the contrary, fitness costs can also be absent as seen in the house fly, *Musca domestica*, where a fitness advantage was observed for *kdr* mutatation in the voltage-gated sodium channel (Rinkevich *et al.* 2013).

Zhang *et al.* (2015) investigated the temperature effects on organophosphate (OP) resistant DBM strains and concluded that extremely cold or warm conditions are unfavourable for insect development and may also modify the fitness costs associated with pesticide resistance. They found that the fitness costs associated with the *ace1R* allele which is causing OP resistance were greater at high temperatures. On the contrary, low fitness caused by high temperatures can affect the resistance under high temperatures, as shown in a methamidophos resistant DBM strain (Liu *et al.* 2008). However, our understanding of how temperature affects the dynamics of pesticide resistance is fairly limited due to the scarcity of data.

Here, we investigated the effect of temperature on the fitness of three resistant strains of *P. xylostella* in comparison to a susceptible reference strain. Generally, it is well known that alleles which confer a higher adaptive value in one environment may have a negative impact on fitness in another environment (Zhang *et al.* 2015). Therefore, our aim was to evaluate changes in the overall fitness, including development, fecundity, mortality and population growth parameters as fitness costs might be involved in DBM resistance against insecticides and rather apparent at higher or lower temperature than optimum temperatures. Furthermore, this study contributes to the understanding of fitness costs in relation to different resistance traits present in DBM and how they possibly can affect the population under applied conditions.

5.2 Material and methods

5.2.1 Insects

The susceptible reference strain BCS-S of *P. xylostella* has been maintained in the laboratory for more than 20 years without any exposure to insecticides. The diamide resistant Philippine strain Sudlon was recently described to be homozygous for a single point mutation in the ryanodine receptor (RyR) gene, resulting in a G4946E amino acid substitution in the transmembrane region of the RyR channel protein (Troczka *et al.* 2012, Steinbach *et al.* 2015). Strain Sudlon-Tfm was obtained by selecting strain Sudlon for ten generations with the benzoylurea insecticide (BPU) triflumuron by incrementally increasing its concentration up to 1000 mg L^{-1}. BPU cross-resistance in strain Sudlon-Tfm was recently linked to a single point mutation from isoleucine to methionine at position 1042 in chitin synthase 1 (Douris, Steinbach *et al.* 2016). The DBM strain Japan was collected from fields with reported field failures of organophosphates (OP) and BPUs in Mizobe, Japan in 2010. We identified mutation I1042M in CHS1 (Douris, Steinbach *et al.* 2016) which was present heterozygously on a low frequency of 20 % in the population (data not shown) but we did not test for OP resistance. All strains were reared on cabbage (*Brassica oleracea*, L.) in different rearing rooms as recently described (Douris, Steinbach *et al.*, 2016). Adults of *P. xylostella* were fed with 10 % (w/v) sucrose solution.

5.2.2 Bioassay method

Leaf dip bioassays were conducted according to IRAC Method No. 7 (www.irac-online.org). We tested a broad range of insecticides including fipronil, deltamethrin, acetamiprid, *B. thuringiensis var. kurstaki*, triflumuron, methoxyfenozide, tebufenozide, indoxacarb, metaflumizone, ryanodine and chlorantraniliprole. The technical grade insecticides (purity >97 %) were prepared as described in Steinbach *et al.* (2015) and obtained in house (Bayer AG, Monheim), except for *Bacillus thuringiensis*. Here, we used Dipel® ES (*B. thuringiensis*

subspecies *kurstaki*, strain ABTS-351, strain HD-1) (Cheminova Deutschland GmbH, Germany) in aqueous dilutions ranging from 1000 to 0.01 mg L^{-1}.

The affected larvae, i.e. those showing symptoms of poisoning and death, were assessed after 96 h for triflumuron and after 72 h for all other insecticides tested. Larvae exposed to aqueous triton X-100-treated leaves served as control and showed a mortality of less than 10% in all bioassays. LC_{50}-values and 95% confidence limits were calculated using Graph Pad Prism 5.03 (GraphPad Software, Inc., USA).

5.2.3 Life tables and experimental conditions

Life time table parameters were investigated at three different temperatures: 20 °C ± 1 °C, 25 °C ± 1 °C and 30 °C ± 1 °C. The strains were placed in plastic cages in a climate chamber and kept under standard conditions at the respective temperature (20 °C, 25 °C or 30 °C), relative humidity 50 % ± 10 %, LD: 16:8 for one generation prior to testing to adapt to the conditions as described previously (Golizadeh *et al.* 2009, Jha *et al.* 2012) .

In order to investigate the ontogenesis from egg to adult, 50 adults of each strain were transferred to a cage and females were allowed to lay eggs for 6 h. Afterwards the cabbage plants were removed and egg development was monitored. Fifty newly hatched first instar larvae (i.e. 50 replicates) were transferred to a 6-well plate containing a wetted filter paper and a cabbage leaf disc (2 cm diameter) which was renewed every day. Development of each replicate was recorded every 6 hours until pupation. Mortality was recorded for every development stage. The experiment at 30 °C was repeated two times as the mortality rate was fairly high.

5.2.4 Adult Longevity, female oviposition and fecundity

Adult longevity, female oviposition (fertility) and fecundity was investigated by maintaining 11 pairs of male and female moths of each strain together in individual rearing cages at the respective temperature under standard conditions. The number of eggs that were laid per female was counted every 12 h. The experiments were continued until the last female moth died. All hatched first instar larvae were collected and maintained on cabbage until adults emerged. Then the sex was determined and recorded for sex ratio calculation.

5.2.5 Life and Fertility tables

Population growth parameters, net reproductive rate (R_0), intrinsic rate of natural increase (r_m), mean generation time (*T*), doubling time (*DT*), and finite rate of increase (λ) were calculated using the following equations described in Krebs (1994):

The net reproductive rate (R_0) is equal to the sum of the $l_x m_x$ products as in the following equation: $R_0 = \sum l_x m_x$, where l_x is the age-specific survival rate which is the proportion

surviving to each life stage ($l_x = \frac{a_x}{a_0}$, calculated by dividing the number of living individuals at the beginning of the stage, a_x, by the initial number of the cohort, a_0) and m_x is the fecundity rate (number of females produced per female).

The cohort generation time (*T*) (in days) was approximated by the following formula: $T = (\sum x l_x m_x) / R_0$ with *X* as the pivotal age for the age class in units of time (days).

The intrinsic rate of natural increase (r_m): $r_m = \ln(R_0)/T$

Doubling time (*DT*), in days, was calculated using: $DT = \ln(2)/r_m$

The finite rate of increase (λ), the number of female offspring per female per day: $\lambda = R_0^{1/T}$

5.2.6 Data analysis

In order to determine the similarities or significant differences between the strains and temperatures, all parameters determined for the four strains of *P. xylostella* were analyzed with one way ANOVA followed by Tukey posthoc test using GraphPad Prism 5.03 (GraphPad Software Inc., USA). Data were checked for normality prior to analysis.

5.3 Results

5.3.1 Bioassays and insecticide cross-resistance

Log-dose probit-mortality data obtained from leaf dip bioassays with third instar DBM larvae of strains BCS-S, Japan and Sudlon are given in Table 1. The diamide resistant strain Sudlon shows high resistance against chlorantraniliprole, deltamethrin, indoxacarb and acetamiprid with resistance ratios (RR) of >10,000, >2000, >100 and 35, respectively. Furthermore, it exhibits moderate resistance levels against fipronil (RR 23) and both ecdysone agonists, methoxyfenozide and tebufenozide (RR >7), but virtually no resistance against metaflumizone and *Bt.*. Strain Japan has shown low to moderate resistance against acetamiprid (RR 5), indoxacarb (RR 7) and deltamethrin (RR 33). Strain Sudlon-Tfm obtained after selection of strain Sudlon exhibited high resistance levels against triflumuron (RR >1000) and other BPU insecticides (Douris, Steinbach *et al.*, 2016).

Table 1. Log-dose probit-mortality data for several insecticides tested against third instar larvae of different strains of diamondback moth in leaf-dip bioassays (72 h and 96 h).

Compound	Strain	n	LC$_{50}$ (mg L^{-1}) (CL95%*)	SLOPE	RR**
Fipronil	BCS-S	240	0.30 (0.25-0.36)	1.89	
	Japan	210	0.52 (0.42-0.65)	1.29	2
	Sudlon	210	6.80 (5.90-7.70)	2.16	23
Deltamethrin	BCS-S	240	0.43 (0.17-1.10)	0.78	
	Japan	240	14 (9.6-19)	1.30	33
	Sudlon	240	>1000		>2300
Acetamiprid	BCS-S	210	1.50 (1.30-1.80)	2.10	
	Japan	180	7.10 (5.70-8.80)	2.10	5
	Sudlon	150	53 (0.58-4915)	3.00	35
B. thuringiensis	BCS-S	210	0.18 (0.07-0.46)	1.60	
var. kurstaki	Japan	210	0.40 (0.15-1.10)	0.72	2
	Sudlon	210	0.56 (0.06-5.60)	0.63	3
Triflumuron[a]	BCS-S	420	5.3 (4.2-6.9)	1.10	
	Japan	420	12 (7.80-17.30)	0.89	2
	Sudlon	420	18 (10-29)	0.90	3
	Sudlon-Tfm	180	>1000		>188
Methoxyfenozide	BCS-S	360	9.40 (8.00-11)	1.70	
	Japan	360	12 (8.10-17)	1.50	1
	Sudlon	300	>200		>21
Tebufenozide	BCS-S	180	31 (28-33)	2.10	
	Japan	180	29 (27-31)	2.10	1
	Sudlon	180	>200		>7
Indoxacarb	BCS-S	180	0.60 (0.43-0.95)	2.10	
	Japan	180	3.90 (2.00-7.90)	1.40	7
	Sudlon	150	63 (0.95-4136)	1.50	105
Metaflumizone	BCS-S	210	0.70 (0.64-0.78)	1.60	
	Japan	210	0.60 (0.59-0.61)	2.20	1
	Sudlon	210	2.00 (1.30-3.10)	1.30	3
Ryanodine	BCS-S	240	1.00 (0.83- 1.20)	1.52	
	Japan	210	1.20 (1.10-1.40)	2.07	1
	Sudlon	210	1.50 (1.40-1.60)	2.58	2
Chlorantraniliprole[b]	BCS-S	480	0.02 (0.013-0.031)	0.75	
	Japan	480	0.045 (0.032-0.064)	0.75	2.3
	Sudlon	180	> 1000		>10000

*95% confidence limits.

**Resistance ratio (LC$_{50}$ of resistant strains divided by LC$_{50}$ of strain BCS-S).

[a]Data taken from Douris, Steinbach *et al.* (2016).

[b]Data taken from Steinbach *et al.* (2015).

5.3.2 Development time, larval weight and mortality

Table 2 shows the duration of different immature stages and total development time of the four strains of *P. xylostella* at three different temperatures (20 °C ± 1 °C, 25 °C ± 1 °C and 30 °C ± 1 °C). The egg duration varied among the strains but there was no significant difference observed

($P > 0.05$; df = 3). Egg development ranged from 3.31 days (BCS-S) to 3.36 days (Sudlon-Tfm) at 20 °C, from 2.81 days (BCS-S) to 2.87 days (Sudlon) at 25 °C and from 2.63 days (BCS-S) to 2.68 (Sudlon-Tfm) at 30 °C. Larval development, from first to fourth instar, was significantly longer in the triflumuron resistant strain Sudlon-Tfm at 20 °C in comparison to the other strains (ANOVA,

$p < 0.005$, F > 30, df = 3). Similar effects were seen at 25 °C and 30 °C although they were not persistent throughout all larval stages. No significant differences were found in prepupa stage; except for 20 °C where the prepupa stage lasted significantly longer in strain Sudlon-Tfm than in its parental strain Sudlon (ANOVA, $p < 0.005$, F > 5, df = 3). The pupa duration varied greatly among the strains at the three temperatures. Strain BCS-S had shown the shortest pupal period at 20 °C of all strains (ANOVA, $p < 0.005$, F > 28, df = 3), whereas strain Sudlon-Tfm exhibited the longest pupation period at 25 °C (ANOVA, $p < 0.05$, F > 3, df = 3). Both strains, Sudlon and Sudlon-Tfm, had a longer pupal period than BCS-S at 30 °C (ANOVA, $p < 0.05$, F > 11, df = 3). Additionally, the total developmental time, from egg to adult, of strain Sudlon-Tfm was significantly increased in comparison to the other strains at all three temperatures. The susceptible reference strain BCS-S exhibited the shortest total developmental time (ANOVA, $p < 0.005$, F > 34, df = 3). Furthermore, the temperature had a significant effect on the life cycle of all strains as the developmental time was reduced with increased temperatures (ANOVA, $p < 0.005$, F > 200, df = 2).

The age-specific mortality rate (q_x) of *P. xylostella* in the four different cohorts showed a similar pattern, including high mortality occurring during egg stage and larval stages (supplementary data Appendix C: Table S1-S4) with no significant difference among the strains (ANOVA, $p > 0.05$,

F = 0.2, df = 3). The survival was high in pupa stage at 20 °C and 25 °C in all strains but considerably lower at 30 °C (Table 3). In addition, the survival of larva and pupa was significantly reduced at 30 °C with a higher mortality rate in the cohorts in comparison to 20 °C and 25 °C (ANOVA, $p < 0.005$, F > 13, df = 2).

There was considerable variation between the strains regarding the weight of immature stages at 25 °C (Table 4). Generally, strains Sudlon and Sudlon-Tfm were of lower weight compared to the other two strains, which was especially prominent in fourth instar and pupa stages (ANOVA,

$p < 0.005$, F > 40, df = 3).

Table 2. Development time (days ± SE) of immature stages of the four strains of *P. xylostella* at three temperatures.

		20 °C		
Stage	**BCS-S**	**Japan**	**Sudlon**	**Sudlon-Tfm**
Egg	3.31 (±0.015) a	3.33 (±0.017) a	3.35 (±0.18) a	3.36 (±0.18) a
Instar I	1.51 (±0.0048) a	1.54 (±0.0060) b	1.61 (±0.0069) c	1.65 (±0.0046) c
Instar II	1.92 (±0.0070) a	1.93 (±0.0065) a	1.94 (±0.011) a	2.02 (±0.0072) b
Instar III	1.90 (± 0.0048) a	1.94 (± 0.0053) b	1.97 (±0.0075) c	2.03 (±0.0087) d
Instar IV	2.42 (±0.0082) a	2.5 (±0.015) b	2.55 (±0.0065) c	2.61 (±0.0074) d
Prepupa	0.78 (±0.011) ab	0.79 (±0.013) ab	0.77 (±0.0094) a	0.83 (±0.015) b
Pupa	4.3 (±0.073) a	4.89 (±0.061) b	4.95 (±0.082) b	5.03 (±0.067) b
Total	16.14 (±0.075) a	16.93 (±0.072) b	17.17 (±0.086) b	17.54 (±0.078) c
		25 °C		
Stage	**BCS-S**	**Japan**	**Sudlon**	**Sudlon-Tfm**
Egg	2.81 (±0.021) a	2.80 (±0.24) a	2.87 (±0.022) a	2.88 (±0.018) a
Instar I	1.39 (±0.022) a	1.40 (±0.026) a	1.43 (±0.020) a	1.45 (±0.018) a
Instar II	1.27 (±0.044) a	1.40 (±0.041) ab	1.37 (±0.021) ab	1.44 (±0.024) b
Instar III	1.15 (±0.043) a	1.16 (±0.029) a	1.30 (±0.041) a	1.47 (±0.034) b
Instar IV	1.45 (±0.021) a	1.36 (±0.028) a	1.59 (±0.023) b	1.88 (±0.042) c
Prepupa	0.63 (±0.020) a	0.59 (±0.021) a	0.64 (±0.020) a	0.66 (±0.023) a
Pupa	3.53 (±0.080) a	3.46 (±0.071) a	3.49 (±0.099) a	3.78 (±0.054) b
Total	12.28 (±0.083) a	12.20 (±0.11) a	12.70 (±0.13) b	13.53 (±0.081) c
		30 °C		
Stage	**BCS-S**	**Japan**	**Sudlon**	**Sudlon-Tfm**
Egg	2.63 (±0.020) a	2.70 (±0.022) a	2.65 (±0.020) a	2.68 (±0.022) a
Instar I	1.00 (±0.015) a	1.03 (±0.017) a	1.04 (±0.015) a	1.06 (±0.014) a
Instar II	1.00 (±0.012) a	1.07 (±0.018) ab	1.09 (±0.016) b	1.11 (±0.014) b
Instar III	0.95 (±0.015) a	1.075 (±0.019) b	1.05 (±0.011) b	1.11 (±0.0099) b
Instar IV	1.04 (±0.012) a	1.08 (±0.012) ab	1.13 (±0.014) bc	1.16 (±0.013) c
Prepupa	0.47 (±0.0081) a	0.51 (±0.013) a	0.51 (±0.0071) a	0.54 (±0.017) a
Pupa	3.15 (±0.042) a	3.22 (±0.052) ab	3.40 (±0.047) bc	3.52 (±0.054) c
Total	10.22 (±0.065) a	10.74 (±0.075) b	10.87 (±0.067) b	11.20 (±0.067) c

Means followed by the same lower case letter within the same row and temperature are not significantly different (P>0.05; Tukey); means followed by a different lower case letter are significantly different (P<0.05, ANOVA, Tukey, df = 3).

Table 3. Within-stage survival (in %) of immature stages of different *P. xylostella* strains at three temperatures.

Temperature	Strain	Egg	Instar I	Instar II	Instar III	Instar IV	Pre-pupa	Pupa
20 °C	BCS-S	96	92	98	96	95	98	100
	Japan	92	98	94	96	95	95	98
	Sudlon	94	92	96	95	98	95	100
	Sudlon-Tfm	94	94	96	98	98	93	98
25 °C	BCS-S	90	94	96	98	95	98	95
	Japan	94	94	94	98	95	95	97
	Sudlon	92	92	93	95	98	98	100
	Sudlon-Tfm	96	96	98	94	95	95	98
30 °C*	BCS-S	84	86	84	86	90	91	67
	Japan	82	87	85	88	89	90	65
	Sudlon	85	87	90	88	91	89	77
	Sudlon-Tfm	85	89	87	87	91	92	77

No significant differences between the strains at life stages (p >0.05, Tukey).
*Survival at 30 °C is significantly different from 20 °C and 25 °C in each strain, p <0.005, ANOVA, F > 13, the experiment was replicated twice.

Table 4. Analyses of wet weight (in mg) of immature stages of *P. xylostella* strains measured at 25 °C. Data are mean values ± SE (n = 50).

	Strain			
Stage	BCS-S	Japan	Sudlon	Sudlon-Tfm
Instar I	0.019 (±0.00029) a	0.018 (±0.00032) b*	0.017 (±0.00034) b*	0.019 (±0.00045) a
Instar II	0.103 (±0.0027) a	0.198 (±0.0056) b	0.059 (±0.0086) c	0.064 (±0.013) ac
Instar III	0.443 (±0.011) a	0.502 (±0.019) b	0.418 (±0.018) a	0.454 (±0.018) a
Instar IV	1.82 (±0.024) a	1.69 (±0.032) b	1.47 (±0.034) c	1.43 (±0.011) c
Pupa	7.23 (± 0.14) a	6.82 (±0.15) a	4.44 (±0.13) b	5.23 (±0.16) c

*Means significantly different (p<0.05, ANOVA, Tukey, df = 3).
Means in rows followed by different letters indicate significant differences between strains at p<0.005, ANOVA, Tukey, df = 3.

5.3.3 Oviposition, fecundity, adult longevity and sex ratio

Oviposition period, fecundity and adult longevity of the four strains of *P. xylostella* are summarized in Table 6. Females of BCS-S strain had a significantly higher fecundity with a mean potential of >210 eggs per female at both 20 °C and 25 °C in comparison to the other strains (ANOVA, p < 0.005, F = 11, df = 3). There was a significant reduction in fecundity at 30 °C for all four strains of *P. xylostella* compared to 20 °C and 25 °C (ANOVA, p < 0.005, F > 80, df = 2).

The trend of age-specific fecundity (m_x) showed that the first eggs were laid at day 18 at 20 °C (BCS-S), which was one day earlier than for strain Japan and strain Sudlon and two days for

strain Sudlon-Tfm (Figure 1). The reproduction started at a similar time for all strains at 25 °C (day 15) and 30 °C (day 12) except for strain Sudlon-Tfm, where oviposition started one day later. A distinct peak of m_x showed a rapid initial increase of eggs laid in all strains for the three temperatures. Thus, most of the eggs were laid in the early days of adult emergence and the numbers decreased rapidly in the second half of adult life span. The pre-oviposition and oviposition period was similar between the strains at the three temperatures, ranging from the longest of 1.50 days (Sudlon-Tfm) at 20 °C to 0.61 days at 30 °C (Sudlon) and 10 days at 20 °C (Sudlon) to 5.48 days at 30 °C (Japan), respectively (Table 6). Strain BCS-S exhibited a significantly shorter post-oviposition period of 2.18 days at 20 °C in comparison to the other strains (ANOVA, $p < 0.005$, $F > 6$, $df = 3$). The increase of temperature resulted in a significant decrease in the overall oviposition period (ANOVA, $p < 0.005$, $F > 20$, $df = 2$). No temperature effects or differences between the strains were observed regarding the sex ratio (Table 5). The sex ratio was nearly 1:1 for the strains at the three temperatures revealing an equal proportion of females and males.

Adult longevity was greatly influenced by temperature in both females and males in all strains of DBM (ANOVA females: $p < 0.005$, $F > 160$, $df = 2$; males: $p < 0.005$, $F > 60$, $df = 2$). In general, adult males tended to die approximately one day earlier than females. Furthermore, the longevity of BCS-S females was significantly shorter compared to the other strains (ANOVA, $p < 0.005$, $F > 26$, $df = 3$). Such an effect was not observed in BCS-S males, except at 20 °C (ANOVA, $p < 0.005$, $F > 54$, $df = 3$). For additional data such as age-specific survival rate (l_x) and life expectancy (ex) see supplementary Appendix C: Tables S1-S4.

Table 5. Sex ratio (female/male ±SE) (n ≥ 350) of different strains of *P. xylostella* at three different temperatures.

Temperature	Strain			
	BCS-S	**Japanese**	**Sudlon**	**Sudlon-Tfm**
20 °C	1.1 (±0.034)	1.1 (±0.037)	1.0 (±0.023)	1.0 (±0.048)
25 °C	1.0 (±0.047)	1.0 (±0.016)	1.0 (±0.031)	1.1 (±0.026)
30 °C	0.98 (±0.046)	1.0 (±0.041)	1.0 (±0.048)	1.0 (±0.033)

Table 6. Oviposition period, adult longevity (days) and fecundity (eggs per female) of different strains of *P. xylostella* at three temperatures. Data are mean values ± SE (n=11).

Temperature	Strain	Oviposition periods			Fecundity	Longevity	
		Pre-oviposition	Oviposition	Post-oviposition	No. of eggs/female	Female	Male
20 °C	BCS-S	1.55 (±0.16) a	8.91 (±0.28) a	2.18 (±0.30) a	214.20 (±4.59) a	12.64 (±0.20) a	12.00 (±0.23) a
	Japan	1.46 (±0.16) a	9.46 (±0.28) a	4.18 (±0.30) b	172.40 (±7.33) b	15.09 (±0.25) b	14.00 (±0.30) ab
	Sudlon	1.55 (±0.16) a	10.00 (±0.19) a	4.64 (±0.28) b	180.30 (±4.41) b	16.18 (±0.23) b	15.64 (±0.41) b
	Sudlon-Tfm	1.50 (±0.15) a	9.75 (±0.28) a	4.33 (±0.36) b	175.60 (±6.17) b	15.58 (±0.43) b	14.09 (±0.64) b
25 °C	BCS-S	0.71 (±0.057) a	10.02 (±0.31) a	1.64 (±0.15) a	222.90 (±9.09) a	12.36 (±0.28) a	12.27 (±0.68) a
	Japan	0.75 (±0.048) a	10.07 (±0.41) a	2.46 (±0.31) ab	188.90 (±9.33) ab	13.27 (±0.47) b	12.55 (±0.79) a
	Sudlon	0.80 (±0.057) a	9.93 (±0.35) a	3.55 (±0.31) b	185.90 (±4.44) b	14.27 (±0.51) ab	12.36 (±0.79) a
	Sudlon-Tfm	0.84 (±0.051) a	10.16 (±0.36) a	2.82 (±0.38) ab	171.90 (±5.39) b	13.83 (±0.42) ab	13.09 (±0.49) a
30 °C	BCS-S	0.5 a	4.77 (±0.14) a	0.27 (±0.14) a	104.10 (±3.90) a	5.55 (±0.21) a	5.00 (±0.30) a
	Japan	0.61 (±0.040) a	5.48 (±0.16) a	1.00 (±0.23) ab	89.18 (±3.31) b	7.09 (±0.21) b	6.36 (±0.31) a
	Sudlon	0.61 (±0.040) a	4.57 (±0.21) a	1.18 (±0.12) b	101.00 (±2.49) ab	7.18 (±0.23) b	6.18 (±0.40) a
	Sudlon-Tfm	0.59 (±0.038) a	5.32 (±0.090) a	0.55 (±0.15) ab	99.33 (±1.63) ab	6.58 (±0.23) ab	6.00 (±0.36) a

Means in columns at the same temperature followed by different letters indicate significant differences (ANOVA, $P<0.005$, Tukey, df = 3).
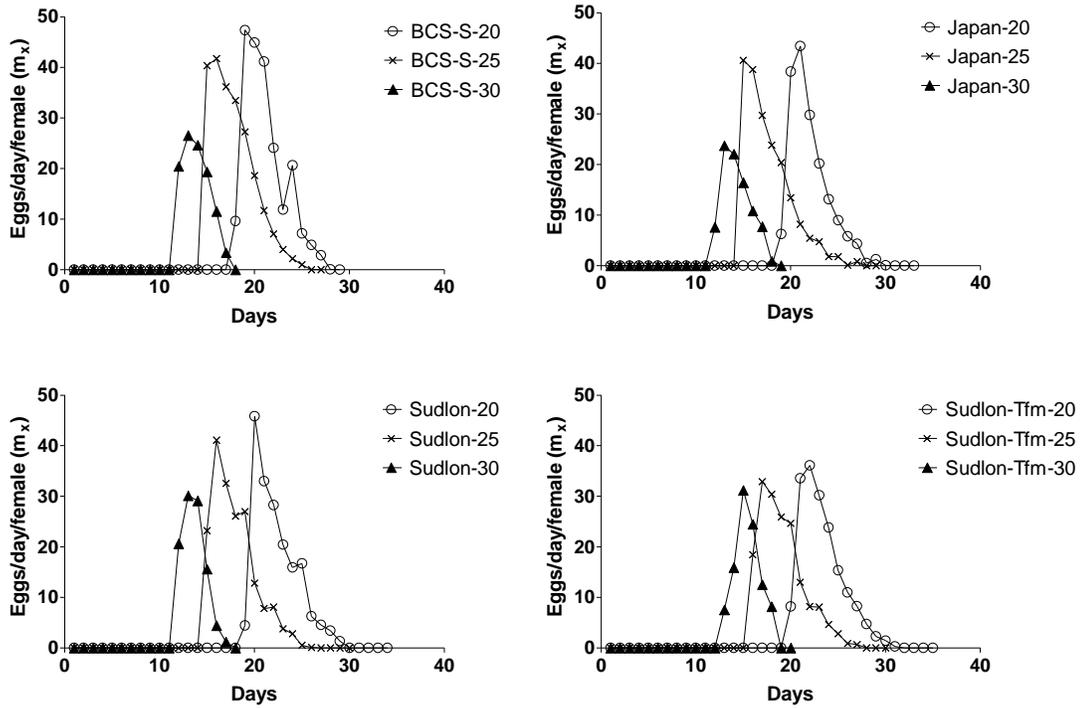
Figure 1. Daily oviposition curves (fecundity, m_x = eggs/day/female) (n = 11) of females of different strains of *P. xylostella* at three different temperatures.

5.3.4 Population growth parameters

The population and reproductive parameters of the four strains of *P. xylostella* at the three temperatures are summarised in Table 7. The net reproductive rate (R_0) of the cohorts was highest in the sensitive strain BCS-S, i.e. 77.89 at 20 °C, 86.93 at 25 °C and 8.50 at 30 °C, in comparison to the other strains. The lowest R_0 was observed in strain Sudlon-Tfm with 68.48 female offspring born at 20 °C, 60.95 at 25 °C and 5.95 at 35°C, respectively. The R_0 for strain Japan and Sudlon was found in between BCS-S and Sudlon-Tfm. Similar results were obtained for the intrinsic rate of natural increase (r_m) and the daily finite rate of increase (λ). Where strain BCS-S had shown the highest r_m per female per day of 0.27 at 20°C, 0.37 at 25 °C, 0.21 at 30 °C and the highest finite rate of increase of 1.31 female offspring per female per day at 20°C, 1.44 at 25°C, 1.23 at 30°C. These parameters however, indicate a growth in population size for all the strains tested. The doubling time (*DT*) was shortest in strain BCS-S at all temperatures whereas strain Sudlon-Tfm displayed the longest doubling time at all tested temperatures in comparison to the other strains. The same was found for the mean generation time (*T*). In general 25 °C appears to be the optimum temperature for population growth under the chosen bioassay conditions.

Table 7. Life table statistics (population and reproductive parameters) for different strains of *P. xylostella* at three different temperatures.

Temperature	Strain	Parameter					
		R0	Tc	T	r_m	DT	λ
20 °C	BCS-S	77.89	16.14	16.14	0.27	2.57	1.31
	Japan	61.12	16.92	16.92	0.24	2.85	1.28
	Sudlon	70.32	17.14	17.14	0.25	2.79	1.28
	Sudlon-Tfm	68.48	17.53	17.53	0.24	2.87	1.27
25 °C	BCS-S	86.93	12.23	12.23	0.37	1.90	1.44
	Japan	71.78	12.17	12.17	0.35	1.97	1.42
	Sudlon	72.50	12.69	12.69	0.34	2.05	1.40
	Sudlon-Tfm	60.95	13.56	13.56	0.30	2.29	1.35
30 °C	BCS-S	8.50	10.24	10.24	0.21	3.32	1.23
	Japan	6.69	10.69	10.68	0.18	3.90	1.19
	Sudlon	6.57	10.87	10.87	0.17	4.00	1.19
	Sudlon-Tfm	5.96	11.18	11.18	0.16	4.34	1.17

R0 = Net reproduction rate.
Tc = Approximate generation time, days.
T = Mean generation time (corrected), days.
r_m = Intrinsic rate of increase, day/1.
DT = Doubling time, days.
λ = Finite rate of increase.

5.4 Discussion

DBM has developed resistance against many different chemical classes of insecticides, such as organophosphates, pyrethroids, benzoylureas, abamectin, indoxacarb (Oliveira *et al.* 2011, Santos *et al.* 2011), and *B. thuringiensis* (*Bt*) (Zago *et al.* 2014). However, most recently DBM has evolved high levels of resistance against diamide insecticides such as chlorantraniliprole and flubendiamide due to the extensive use of this new class in different geographies (Troczka *et al.* 2012, Wang and Wu 2012, Ribeiro *et al.* 2014, Steinbach *et al.* 2015). Here, we have investigated the cross-resistance profile of the diamide resistant DBM strain Sudlon recently collected in the Philippines (Steinbach *et al.* 2015) and an OP resistant strain from Japan in comparison to an insecticide susceptible reference strain (BCS-S). Strain Sudlon exhibited varying levels of resistance against all classes of insecticides tested when compared to strain BCS-S, most likely due to recent selection with different mode of action classes under applied conditions. The cross-resistance pattern clearly demonstrates its propensity to develop resistance upon selection and to maintain the alleles that confer resistance within the population for many generations even without further selection pressure. This is a major problem for IPM as it limits the chemical measures to control this pest over several generations due to the persistence of certain resistance alleles at low frequency.

High levels of resistance are usually associated with target-site mutations as demonstrated for several modes action in DBM (Tang *et al.* 1997, Schuler *et al.* 1998, Troczka *et al.* 2012, Ribeiro *et al.* 2014, Douris, Steinbach *et al.* 2016). However, diamide insecticide resistance in strain Sudlon, which is conferred by an amino acid substitution (G4946E) in the ryanodine receptor trans-membrane domain (Troczka *et al.* 2012), has been shown quite stable as the strain maintained its high resistance levels against diamides for more than 50 generations in the laboratory without any selection pressure (Steinbach *et al.* 2015). By selecting strain Sudlon with triflumuron, a BPU insecticide inhibiting chitin biosynthesis, we obtained a homogenous BPU resistant strain (Sudlon-Tfm) carrying a point mutation (I1042M) in chitin synthase 1 as recently described (Douris, Steinbach *et al.* 2016). Furthermore, the homozygously resistant strain Sudlon-Tfm also retained its BPU resistance (and diamide resistance) without any further selection (data unpublished). In addition, we identified two mutations, L1014F (*kdr*) and T929I, in the voltage-gated sodium channel of strain Sudlon which confer pyrethroid (deltamethrin) resistance (data not shown) similar to that which was published by Sonoda *et al.* (2012). To the best of our knowledge, no fitness costs have been reported so far concerning these mutations in DBM.

Here, we compared three resistant DBM strains with a susceptible reference strain to check for possible negative trade-offs/fitness costs concurrently associated with the alleles that confer target-site resistance against diamides (strain Sudlon) and BPUs in addition to diamides (Strain Sudlon-Tfm). The total development time of the susceptible reference strain BCS-S was significantly shorter at 20 °C and 30 °C compared to the resistant strains. Moreover, strain Sudlon-Tfm had shown the longest developmental period from egg to adult. Strain Japan was significantly affected by the lower and higher temperature thus leading to a slower overall development in comparison to the optimum temperature at 25 °C, where the development time was not different from strain BCS-S. The weight of the immature stages of strain Sudlon and Sudlon-Tfm was reduced compared to the other two strains. The three resistant strains had shown a reduced fecundity in comparison to BCS-S at the three temperatures. Thus, strain Japan had shown the lowest net reproductive rate (R_0) at 20 °C. Additionally, Sudlon-Tfm displayed the lowest reproduction parameters (R_0, r_m) and the longest generation time as well as doubling time which affects the overall fitness of the strain. No significant differences were observed in the oviposition periods between the strains, except for an elongation in the post-oviposition period in the resistant strains due to longer longevity at 20 °C, 25 °C and 30 °C of the resistant strains compared to BCS-S. It is not clear whether the negative trade-offs found in this study derived from resistance and its pleiotropic effects, or from associated genetic traits or from an interaction between them. Similar observations were made when comparing fitness parameters of two resistant strains (pyrethroid and BPU resistance) of the codling moth *Cydia pomonella* to a susceptible strain which were not derived from the same line. High

fitness costs were observed in both resistant lines resulting in low female fecundity, longer larval developmental times, smaller body mass of resistant instars and shorter adult lifespan of resistant males (Boivin *et al.* 2001). Selection pressure on a small number of insects can result in inbreeding within a population which can cause changes in morphological and life history traits such as extreme deleterious effects, extremely healthy individuals, or a disproportion in the ratio of males to females. Furthermore, inbreeding leads to a loss of genetic diversity and can have negative consequences for the insect population in terms of environmental adaptation. In order to provide information regarding this factor, bottleneck studies need to be conducted which cover the genetics of a population as well as the fitness while selecting for resistance in a specific population.

We have shown in this study that the temperature had significant effects on all four strains which resulted in a reduction of life span, oviposition periods, fertility, development time and also lead to a higher mortality at 30 °C. DBM is able to develop successfully between constant temperatures from 8 to 32°C but extreme temperatures ranging from 4 to 6°C or from 34 to 40°C impaired development or only allowed partial development (Liu *et al*. 2002). Interestingly, no differences were found between populations of *P. xylostella* from tropical and temperate regions of Asia in terms of net reproductive rate and egg production when rearing them at different temperature regimes (Shirai 2000). Li *et al.* (2007) demonstrated that fitness costs were temperature-dependent in a spinosad resistant field strain of *P. xylostella*. The costs were increasing in scale at unfavourably low and high temperatures and were particularly high at low temperatures as DBM failed to reproduce. Temperature effects were also observed in *Copitarsia decolora* (Lepidoptera) influencing fecundity, developmental time and survival (Gould *et al.* 2005). At higher temperatures, the generation time was shorter, but the survival was reduced from the highest survival of 93 % at 24.9 °C to the lowest of 25 % at 29.5 °C. Furthermore, the temperature also affects the host plant which in turn has a consequence on food uptake during the larval period and could thus change adult longevity and oviposition (Awmack and Leather 2002).

Our data clearly indicated that fitness costs were involved in BPU resistance in strain Sudlon-Tfm when directly comparing it to its parental strain Sudlon, which is very likely to share the same genetic background apart from the reported target-site mutation in CHS-1 (Douris, Steinbach *et al.* 2016). However, negative trade-offs were not found in *D. melanogaster*, when the respective mutation (I1042M) conferring BPU resistance in strain Sudlon-Tfm was introduced into the *Drosophila kkv* gene by genome editing (Douris, Steinbach *et al.* 2016). This finding suggests the mutation is possibly not directly causing the observed fitness costs and other mechanisms might play a role such as detrimental genes. We conducted next generation sequencing which revealed differences in the gene expression profile between strain Sudlon and Sudlon-Tfm where different genes coding for metabolic enzymes were up- or down-regulated (data not shown). This leads to the

speculation if metabolic factors might be involved in the observed fitness costs between Sudlon and Sudlon-Tfm. In *Bt* resistant *Trichoplusia ni*, it was found that fitness costs were condition dependent and were greatest in the most stressful environment, however Janmaat and Myers (2005) were unable to discern whether the fitness cost were related to direct effects of the resistance trait on life history (i.e. pleiotropy) or linked to detrimental genes. In order to distinguish these, a more rigorous genetic protocol with repeated introductions of the resistant gene into the same genetic background would be required (Bergelson and Purrington 1996). Previous studies have shown that neither exposure to insecticides nor the expression of resistance genes necessarily negatively impacts the fitness of insects; however both factors can increase or decrease the fitness (Fournier *et al.* 1988, Haubruge and Arnaud 2001, James and Price 2002, Ako *et al.* 2004). A diamide resistant *P. xylostella* from Brazil showed negative biological trade-offs, such as significantly reduced larval weight and fecundity, when being compared with the susceptible individuals in both the absence and presence of chlorantraniliprole (Ribeiro *et al.* 2014). However, Han *et al.* (2012) and Lai and Su (2011) found no significant effect of chlorantraniliprole on the longevity of *P. xylostella* and *S. exigua* adults, respectively, when the larvae were treated with sublethal concentrations of this insecticide, which was probably related to the different age of larvae which were tested in those studies. Raymond *et al.* (2005) have shown that two different *Bt* resistant strains of DBM exhibited fitness costs under different environmental conditions indicating that the interaction between genes and environment rather than the physiological basis of resistance determined the consequences of a novel adaptation for individual insects. Finally, it was recently demonstrated that a cyantraniliprole resistant strain of DBM displayed an overall lower reproductive ability and relative fitness than the two reference strains, i.e. the non-selected population and the susceptible laboratory strain (Liu *et al.* 2015).

In summary, we observed significant fitness costs in strain Sudlon-Tfm, which were found to be highly resistant to both diamide and BPU insecticides when compared to strain Sudlon at all three temperatures. Both strains share the same genetic background therefore; we associate these fitness costs with the genetic processes involved in BPU resistance. Thus the reduced population growth examined in Sudlon-Tfm could have a greater impact on the overall population size in the absence of the insecticide. Furthermore, 30 °C is unfavorable for DBM development as it had a negative impact on the overall fitness of all four strains where the resistant strains were significantly more affected than the susceptible reference strain. Fitness costs were also apparent in strain Japan at 20 °C and 30 °C in comparison to BCS-S which could be related to resistance and stress caused by the low or high temperature. However, strain Sudlon exhibited significant differences at all three temperatures when comparing to BCS-S, hence it is not clear whether the observed effects are caused by resistance or actual variances between strains with a different genetic background.

In conclusion benzoylurea selection pressure on a diamide-resistant *P. xylostella* strain resulted in lowest reproduction parameters as well as both the longest generation time and doubling time among all strains tested. Thus, suggesting significant effects on the overall fitness and population growth parameters for diamide resistant populations pressured by benzoylureas under applied conditions.

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Chapter 6 Concluding discussion

6.1 Resistance

Lepidopteran pests, such as diamondback moth (DBM), *Plutella xylostella* and tomato leaf miner, *Tuta absoluta*, are among the most destructive and invasive species. Without proper plant protection management, mass outbreaks of these pests can cause severe damage to crop production resulting in high yield losses ranging from 50 % up to 100 % for all potential food and fibre crops (Calderon and Hare 1986, Verkerk and Wright 1996, Peshin 2002). In order to limit crop losses, insecticides were introduced to the market offering novel measures to control pest insects. For more than 60 years synthetic insecticides have been used successfully and advances in research have allowed a constant development of new insecticide chemistries with improved target selectivity and specificity. Due to their high efficacy, insecticides have been applied intensively, especially as a single-component strategy in conjunction with frequent sprayings. As a consequence pests have been subjected to strong selection pressures which has prompted the development of insecticide resistance resulting in insecticide field-failures and pest control failures within only a short period of time. Other factors including high application rates, mono-cropping and wrong management are facilitating the spread of resistance genes. Pests like DBM which exhibit a high reproductive potential, a short life cycle with several generations per year and high genetic plasticity (Ulmer *et al.* 2002) are able to literally overcome efficacy of any class of insecticides and are likely to develop multiple strategies to reduce toxicity. The interactions between multiple insecticide resistance loci can shape the effectiveness of chemical control efforts as they can interact in an additive fashion, synergistically or antagonistically (Hardstone and Scott 2010). Additionally, high levels of cross-resistance are often observed between insecticides with a similar mode of action (MoA) which has a major impact on chemical control measures (Pu *et al.* 2010) as persistent applications of insecticides sharing a common biochemical target need to be avoided.

Pest control is complicated by the fact that homozygous and heterozygous resistant genotypes exist in field populations and many resistance alleles are primarily present in hybrids at low frequencies. Thus, the evolution of resistance depends on the pest species, life stage, the insecticide used as control measure, the relative fitness of the hybrid compared with the susceptible genotype and the benefits that are associated with each mechanism of resistance (Roush and McKenzie 1987, Carrière *et al.* 2002). The rapid development of resistance in pest populations, especially against new molecules such as diamides exemplifies the complexity of the dynamics of resistance. Therefore, it is important to assess the mechanisms that underlie resistance in order to implement the best RM strategies in a timely manner to slow down the development and spread of resistance in pests. Without the knowledge of resistance mechanisms, the strategies might be less effective or fail completely.

6.1.1 Diamide Insecticide Resistance

The diamides are the most recent addition to the insecticide market and are highly efficacious in controlling a wide spectrum of pests including Lepidoptera. They exhibit specific target-site activity by acting on the insect ryanodine receptor (RyR) causing calcium depletion from internal stores in muscles leading to insect mortality through starvation and paralysis (Cordova *et al.* 2006). It was shown that diamides bind the RyR with nanomolar affinity to a site different from ryanodine (Cordova *et al.* 2006, Lümmen *et al.* 2007, Qi and Casida 2013), but allosterically coupled and located in the C-terminal membrane-spanning domain (Kato *et al.* 2009, Isaacs *et al.* 2012, Lümmen 2013).

Diamides have risen to a high level of prominence in the marketplace due to their high efficiency as well as favourable biological, ecological and toxicological attributes (Teixeira and Andaloro 2013). In 2014 they captured a large market share of 8 % ($1.4B) and it is estimated that the sales could surpass $2B by the year 2020 as new anthranilic diamides such as cyclaniliprole and tetraniliprole are currently under development (Sparks and Nauen 2015).

The intensive uses of this chemical class, especially as a control measure for DBM, lead to a rapid selection of resistant populations in the field which seriously compromised diamide field efficacy. Thus, first cases of diamide field failures against DBM were reported only 2 years after their introduction to market in the Philippines and Thailand (Troczka *et al.* 2012). Subsequently more reports from other regions such as China (Wang and Wu 2012, Wang *et al.* 2013, Gong *et al.* 2014) and Brazil (Ribeiro *et al.* 2014) followed indicating that the resistance is spreading rapidly on a global scale (Steinbach *et al.* 2015). In addition, other lepidopteran pests have shown diamide tolerances ranging from moderate resistance in rice stem borer, *Chilo suppressalis* (Gao *et al.* 2013, He *et al.* 2014) and beet armyworm, *Spodoptera exigua* (Lai *et al.* 2011, Che *et al.* 2013) up to high resistance ratios in *T. absoluta* (Roditakis *et al.* 2015), and smaller tea tortrix, *Adoxophyes honmai* (Uchiyama and Ozawa 2014). Diamide insecticide resistance in *T. absoluta* was first reported in Sicily, Italy and Greece in 2014 after five years of monitoring pest activity (Roditakis *et al*. 2015).

Troczka *et al.* (2012) identified an amino acid substitution from glycine to glutamic acid at position 4946 in the C-terminal region of the ryanodine receptor (RyR) in highly diamide resistant DBM populations field collected from Philippines (Sudlon) and Thailand. It was shown that the amino acid substitution evolved independently as one strain exhibited non-synonymous single-nucleotide polymorphisms from GGG to GAA (Philippines) and the other one from GGG to GAG (Thailand) while both resulted in a G4946E substitution. The G4946E target-site mutation was also present in DBM populations from China (Gong *et al.* 2014, Guo *et al.* 2014a,b, Yan *et al.* 2014) and Japan (Sonoda *et al.* 2017) showing different levels of resistance to diamide insecticides. The differences in resistance ratios is most likely driven by

the proportion of G4946E homozygous resistant (RR) genotype present in a population as hybrids carrying one resistant and one susceptible allele show a susceptible phenotype which was shown in Chapter 2 by further characterising the G4946E mutation in strain Sudlon derived from the Philippines (Steinbach *et al.* 2015). Only homozygotes exhibiting two resistant alleles can overcome diamide toxicity. The highly diamide resistant strains of DBM which have been examined so far show an autosomal, almost incompletely recessive mode of inheritance (Wang *et al.* 2012, Guo *et al.* 2014b, Steinbach *et al.* 2015) with a nearly monogenic response based on the presence of the homozygous G4946E mutation in the RyR (Chapter 2, Steinbach *et al.* 2015). Genotyping DBM larvae collected from different countries revealed that the G4946E mutation is widespread on a global scale and that the presence of homozygotes RR genotype correlates with diamide insecticide field failure (Chapter 2, Steinbach *et al.* 2015). This supports the hypothesis that the resistance has evolved independently rather than through migration of one population. It is crucial to point out that cross-resistance to phthalic and anthranilic diamides was observed in DBM (Troczka *et al.* 2012, Liu *et al.* 2015b, Chapter 2, Steinbach *et al.* 2015) and other lepidopteran pests such as *A. honmai* (Uchiyama and Ozawa 2014) and *T. absoluta* (Roditakis *et al.* 2015, Chapter 3, Roditakis *et al.* 2017).

The first approaches to provide functional evidence on the implications of a G4946E mutation in DBM RyR by performing fluorescence polarization binding assays, largely failed due to the low specificity of the diamide-like florescent probe with I_{50}-values of greater than 1000 nM (Guo *et al.* 2014a,b). Comparing the binding affinity of the fluorescent tracer to RyR of diamide resistant and susceptible populations, the difference was less than 3-fold which hardly explains the resistance ratios of greater than 1000-fold estimated from bioassays. However, by performing radioligand binding studies using thoracic microsomal membrane (SR/ER) preparations of DBM strain Sudlon, functional evidence was provided for the first time that the G4946E mutation has implications on both diamide-specific binding as well as on its concentration-dependent allosteric modulation of [³H]ryanodine binding (Chapter 2, Steinbach *et al.* 2015). Furthermore, the diamide resistant strain did not show specific saturable binding of the des-methylated flubendiamide analogue [³H]PAD1 whereas the susceptible strain revealed highly specific and saturable binding to RyR obtained from thoracic muscle membranes. Troczka *et al.* (2015) showed that the G4946E mutation in *Plutella* RyR recombinantly expressed in clonal Sf9 cell lines dramatically impaired the binding of both phthalic and anthranilic diamides. The latter named studies underline the importance of the G4946E mutation for diamide resistance.

Recently, Guo *et al.* (2014b) identified three more mutations, E1338D, Q4594L and I4790M, located in the C-terminal end of the RyR of a Chinese DBM strain which was highly chlorantraniliprole resistant. Interestingly, the mutation I4790M described by Guo *et al.* (2014b) is located directly opposite of the G4946E mutation with a distance between the respective Cα

atom positions of the mutation sites of approx. 13 Å as shown in homology models of the diamondback moth RyR based on the recently published closed-state cryo-EM structure of rabbit RyR1 (Yan *et al.* 2015) (Chapter 2, Steinbach *et al.* 2015). The critical role of the transmembrane domain at the interface between helix S4 and the S4–S5 linker for diamide binding seems obvious regarding the functional implications of G4946E in diamide binding (Nauen and Steinbach 2016).

In chapter 3, the discovery of the mutation G4903E in the RyR of the diamide selected *T. absoluta* strain IT-GELA-SD4 derived from Italy was reported which is corresponding to the mutation G4946E in DBM (Roditakis *et al.* 2017). Additionally, strain IT-GELA-SD4 exhibited an alternative amino acid substitution from glycine to valine (V) at the same position, G4903 as well as the mutation I4790M. Radioligand binding studies using microsomes derived from thoracic muscle membrane preparations of diamide susceptible and resistant, IT-GELA-SD4 *T. absoluta* strains revealed that the detected G4903E/V and I4746M RyR have functional implications for diamide binding (Chapter 3, Roditakis *et al.* 2017). Diamide resistance in *T. absoluta* strain IT-GELA-SD4 was autosomally inherited with an incompletely recessive trait which corresponds to the mode of inheritance of diamide resistance mediated by RyR target-site mutations in DBM (Guo *et al.* 2014b, Steinbach *et al.*, 2015, Chapter 3, Roditakis *et al.* 2017).

Interestingly, when genotyping larvae of *T. absoluta* strain IT-GELA-SD4, the presence of the mutation G4903E/V was homozygous RR whereas I4790M was present as homozygous wildtype SS, heterozygous SR or homozygous RR on different ratios (Chapter 3, Roditakis *et al.* 2017). Similar observations were made in two *T. absoluta* strains from Brazil, BR-PSQ and BR-GML1, which had shown target-site mutation G4903E homozygous RR and a novel mutation at position 4790 in the RyR where isoleucine was substituted by tyrosine (Chapter 3, Roditakis *et al.* 2017). However, all strains were highly resistant against chlorantraniliprole but showing different levels of resistance which could be related to the different genetic background of the populations or the experimental procedure.

It was speculated that the I4790 mutation site identified in the RyR of DBM is possibly involved in selectivity issues (Chapter 2, Steinbach *et al.* 2015) which has been observed for anthranilic and phthalic diamides in different insect species with varying receptor sensitivity (Qi and Casida 2013, Qi *et al.* 2014). The importance of the amino acid at position 4790 as a selectivity determinant is best shown in Coleopteran pests as they carry a methionine at position 4790 in the RyR thus flubendiamide is not effective against beetles whereas anthranilic diamides are successfully used to control pests of this insect order.

Following this approach, the proportion of mutation I4790M to G4946E might slightly increase when selecting with chlorantraniliprole instead of flubendiamide as shown in Guo *et al.* (2014b) when DBM was subjected to chlorantraniliprole and had shown an increased ratio of mutation I4790M in comparison to G4946E. In other words, selecting with flubendiamide for diamide

resistance would result in a homozygous population carrying the mutation G4946E as it provides tremendous resistance levels. Furthermore, this could explain the high ratios of target-site mutation G4946E compared to mutation I4790M in *T. absoluta* strains from Brazil, BR-PSQ, BR-DML1, (Chapter 3, Roditakis *et al.* 2017) as flubendiamide has largely been applied to control this pest in Brazil (personal communication with Herbert Siqueira). Sonoda *et al.* (2017) tested flubendiamide against 3rd instar larvae of DBM from 11 populations from Japan and the diamide resistant phenotype was found to be highly correlated with the presence of target-site mutation G4946E whereas the authors did not detect the mutation Q4594L and only limited E1338D and I4790M target-site alterations in DBM.

Studying diamide resistance in DBM it was shown that reversal of resistance may occur in absence of selection pressure (Wang *et al.* 2013, Ribeiro *et al.* 2014). Thus, the possibility of involved fitness costs cannot be excluded as they can play a critical role in the survival and distribution of the mutant allele (see next Chapter 6.2). Another aspect that needs to be considered is the difference in the intrinsic activity of the individual diamide compounds. Cyantraniliprole had shown a higher intrinsic activity in susceptible and resistant DBM strains than other diamide insecticides (Chapter 2, Steinbach *et al.* 2015).

A few cases of metabolic mechanisms involved in diamide resistance have been reported so far, such as in *Choristoneura rosaceana* where a correlation between esterase activity and susceptibility to chlorantraniliprole was found (Sial *et al*. 2011, Sial and Brunner 2012). In a number of field-collected Brazilian strains of *T. absoluta* the level of cytochrome P450 activity was significantly correlated with the variation in chlorantraniliprole and cyantraniliprole susceptibility suggesting that oxidative metabolism plays to some extent a role in diamide resistance (Campos *et al.* 2015). However, the authors did not find such correlation for the observed variation in flubendiamide efficacy. Muthusamy *et al.* 2014 also reported that oxidative metabolism might be involved in diamide resistance in a laboratory-selected strain of *S. litura* from India which had shown a 80-fold resistance to chlorantraniliprole, but synergist studies using piperonyl butoxide (PBO) were not conclusive both *in vitro* and *in vivo*.

In summary, metabolic mechanisms including changes in the activity of esterases or cytochrome P450s as demonstrated might contribute to enhanced detoxification of diamide insecticides but are rather negligible for diamide resistance. This is in contrast to target-site mutations identified in the RyR of DBM and *T. absoluta* which have been shown to confer high levels of diamide tolerance by impairing diamide binding to the RyR. The implications of target-site alterations have also been shown for several other modes of action in DBM (Tang *et al.* 1997, Schuler *et al.* 1998). So far the two mutations, G4946E and I4790M, are the most important for diamide resistance in Lepidoptera whereas E1338D and Q4594L target-site alterations might only play a minor role. However, diamide resistance is spreading at a fast pace, therefore, monitoring pest resistance status on larger scales remains a powerful tool in detecting changes in susceptibility

levels as soon as they occur affording the opportunity to implement new RM strategies (Jiang *et al.* 2015, Roditakis *et al.* 2015).

6.1.2 Benzoylphenyl urea

The first Benzoylphenyl urea (BPU), diflubenzuron, was developed and launched to the market in the 1970's and over the next four plus decades a total of 15 BPUs have been commercialized including triflumuron and flufenoxuron which exhibit high activity against lepidopteran pests. Despite their small market share accounting only for 3 % of the sales in the total global market for insecticides (Sparks and Nauen 2015), they are still widely used, especially as green insecticides in agricultural crop protection, forestry and sanitary insect pest control against flies and mosquitos (Tomlin 2003). BPUs are commonly known to inhibit chitin biosynthesis by interfering with chitin formation thereby causing molting defects and cuticle malformations (van Eck 1979, Merzendorfer *et al.* 2012). However, the exact mode of action (MoA) had remained elusive for more than 40 years and has only recently been resolved. In chapter 4, it was shown that the BPUs act directly on chitin synthase 1 thereby impairing the last step of chitin polymerization (Douris, Steinbach *et al.* 2016).

BPUs show a high pest activity, low toxicity to mammals and predatory insects and therefore, have been used extensively (Syed 1992). The prolific use of BPUs has resulted in strong selection pressure against the pests leading to BPU resistance which spread rapidly and resulted in yield losses of up to 60 % (Moffit *et al.* 1988).

Monitoring results have shown that DBM developed high levels of resistance against a broad range of BPUs including chlorfluazuron, teflubenzuron within a short period of time (Cheng *et al.* 1988, Cheng *et al.* 1990, Xia et al 2014). Selecting for chlorfluazuron resistance in the laboratory, Fahmy and Miyata (1990) confirmed that DBM is able to develop high levels of resistance (>200 fold) very quickly within only 9 and 10 generations. In the current study, a highly diamide resistant DBM strain (Sudlon) originating in the Philippines with very low resistance ratios against triflumuron was subjected to incrementally increasing concentrations of triflumuron over several generations (Chapter 4, Douris, Steinbach *et al.* 2016). As a result of this, the newly selected strain Sudlon-Tfm was highly triflumuron tolerant with resistance ratios >188 fold and exhibited cross-resistance to all other insect growth regulators (IGRs) tested including flucycloxuron (>1000 fold), lufenuron (196 fold) and diflubenzuron (28 fold). BPUs haven't been used in the Philippines for a long time thus this finding has a direction implication for resistance management. As it is a prime example of resistance mechanisms that are still present in low frequencies in pest species, e.g. DBM and which can become severe when pest populations are subjected to high selection pressure.

Cheng *et al.* (1990) reported a decline in resistance in BPU resistant DBM from 7621- and 243-fold to only 6.5- and 3.1-fold for teflubenzuron and chlorfluazuron, respectively, after 17

selection free generations. However, Sudlon-Tfm maintained a stable phenotype that tolerated high concentrations of BPU without any further selection pressure. In many studies, metabolic resistance was suggested to confer BPU tolerance, such as an increase in microsomal oxidation found in DBM (Perng *et al.* 1988, Lin *et al.* 1989) and *Spodoptera exigua* (Van Laecke and Degheele 1991), or high GST-3 activity shown in DBM (Sonoda and Tsumuki 2005). However, in Chapter 4, a non-synonymous mutation was identified at position 1042 in a highly conserved region in chitin synthase 1 (CHS1) which altered to the amino acid sequence from isoleucine to methionine that conferred BPU resistance in DBM strain Sudlon-Tfm (Douris, Steinbach *et al.* 2016). This target-site mutation, I1042M is located in a highly conserved region in CHSI and at the same position as the mutation I1017F identified in the orthologous gene of the spider mite, *Tetranychus urticae*, conferring resistance to the acaricide etoxazole (van Leeuwen *et al.* 2012). As etoxazole induces moulting defects in fall armyworm, *Spodoptera frugiperda* larvae identical to those caused by BPUs (Nauen and Smagghe 2006), it was assumed that both chemical classes share the same MoA (Douris, Steinbach *et al.* 2016). Introducing either mutation in *Drosophila melanogaster* by a CRISPR/Cas9 coupled with HDR genome modification approach, similar resistance phenotype across different chemical classes of IGRs, such as BPUs, etoxazole, and buprofezin, but not cyromazine were identified.

Genetic changes, such as mutations, conferring resistance might be present in a low frequency in a population and thereby enabling the pest to overcome the toxicity of insecticides. Due to the high genetic plasticity of DBM, this pest is able to develop resistance very quickly. In addition, recent monitoring programs testing the susceptibility values of DBM against chlorfluazuron have shown that populations in China are still susceptible and only three populations have shown a low resistance (Jiang *et al.* 2015). However, owing to the current situation of rapid resistance development, it is highly likely that the tested populations decrease in susceptibility. Therefore, it is essential to apply IPM measures to prevent or delay the evolution of resistance.

Although, metabolic resistance was thought to confer BPU resistance in lepidopteran pests only a few studies confirmed that metabolic processes, such as mixed oxidases or a higher GST-3 activity, are involved. However, the synergistic ratios obtained from these studies were rather low which suggests that metabolic processes play a minor role in BPU resistance. Unlike the newly identified target-site mutation, I1042M, in the CHS1 which's presence was confirmed in DBM strains from India and China. Based on mutation I1042M monitoring programs can be implemented allowing the investigation of resistance status on genetic level in other pests like mosquitoes. Furthermore, this information obtained from the current study allows fresh insight into BPU resistance and the revealed BPU MoA is crucial for resistance management.

6.2 Fitness costs

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The process of natural selection favours genes of phenotypes that show the highest fitness within a population (Holloway *et al.* 1990). As a result of the selection pressure on insects, caused by extensive use of insecticides, the selection of alleles that confer an adaptation to this environmental stress factor is facilitated. Therefore, most insecticide resistance mechanisms are associated with fitness costs as these mutational changes often have deleterious effects on the overall fitness of a resistant insect compared to a susceptible counterpart. However, the costs caused by resistance are not fixed and are more or less dependent on environmental factors, such as temperature (Li *et al.* 2007), food quality (Janmaat and Myers 2005, Golizadeh *et al.* 2009, Farahni *et al.* 2011) and parasitism (Raymond *et al.* 2007). Furthermore, negative genetic trade-offs are often shown in the absence of the insecticide or in the presence of sublethal doses (Hoffmann and Parsons 1991, Ribeiro *et al.* 2014). When selecting for diamide resistance in *P. xylostella*, fitness costs were identified as a consequence of diamide resistance (Han *et al.* 2012, Yan *et al.* 2014), e.g. lower fertility in a cyantraniliprole-selected laboratory strain (Liu *et al.* 2015b). The overall fitness was strongly affected, showing a longer developmental time of larva as well as a decreased rate of pupation and adult emergence with a low relative fitness. When applying a sublethal concentration of chlorantraniliprole to a Brazilian field-evolved chlorantraniliprole-resistant diamondback moth strain (RR >27,000) and a susceptible reference strain, both strains were significantly affected in their fitness (Ribeiro *et al.* 2014). Moreover, the resistant strain had shown negative trade-offs, such as significantly reduced larval weight and fecundity, when chlorantraniliprole was absent. In other studies there was no significant effect on the longevity in *P. xylostella* and *S. exigua* when the insects were treated with a sublethal concentration of chlorantraniliprole (Lai *et al.* 2011, Han *et al.* 2012). In *Cydia pomonella*, it was shown that chlorantraniliprole exposure affected males more than females in terms of mating behaviour (Knight and Flexner 2007). Despite the fitness costs involved in diamide resistance, positive traits could be observed in diamondback moth, such as an increased larval survival, egg hatchability and male longevity (Ribeiro *et al.* 2014). This suggests that a

physiological mechanism is present in order to compensate for associated fitness costs. However, Ribeiro *et al.* (2014) associated the reduced fitness in diamondback moth with the reversion of resistance to chlorantraniliprole as the resistant strain had shown a rapid decline in resistance without selection pressure. Most studies on fitness costs were yet conducted with diamide-resistant diamondback moth strains due to the fact that in most other lepidopteran targeted by diamides, resistance ratios so far reported are quite low and in most cases not compromising field efficacy.

In Chapter 5, it was shown that 30 °C is unfavorable for DBM development as it had a negative impact on the overall fitness of all four strains with the resistant strains being significantly more affected than the susceptible reference strain. In general, the three resistant strains exhibited a significantly longer total development in comparison with strain BCS-S, whereas strain Sudlon-Tfm had shown the longest developmental period from egg to adult. The weight of the immature stages of strain Sudlon and Sudlon-Tfm were reduced compared to the other two strains. Fitness costs were identified in strain Sudlon-Tfm in comparison to strain Sudlon and BCS-S where the overall fitness of strain Sudlon-Tfm was negatively affected showing reduced reproduction parameters (R_0, r_m) and significantly longer generation time at all three temperatures tested (i.e. 20 °C, 25 °C and 30 °C). Thus, the observed fitness disadvantages in strain Sudlon-Tfm might be caused by BPU resistance as both strains, Sudlon and Sudlon-Tfm, are likely to share the same genetic background except for the reported target-site mutation in CHS-1 (Chapter 4, Douris, Steinbach *et al.* 2016). It is important to point out that negative trade-offs were not found in *D. melanogaster*, when the respective mutation (I1042M) conferring BPU resistance in strain Sudlon-Tfm was introduced into the *Drosophila kkv* gene by genome editing (Douris, Steinbach *et al.* 2016). This finding suggests that other mechanisms such as detrimental genes might be involved. A more rigorous genetic protocol with repeated introductions of the resistance gene into the same genetic background would be required in order to discern if fitness costs are related to direct effects of the resistance trait on life history (i.e. pleiotropy) or linked to detrimental genes (Bergelson and Purrington 1996). However, subjecting a small number of insects to insecticide selection pressure as performed in the laboratory can result in inbreeding within a population. This in turn has negative effects on the overall fitness as it can cause changes in life history traits leading, for example, to extremely healthy individuals, or a disproportion in the ratio of males to females. Furthermore, a population that is inbred shows a reduction in genetic diversity which can have negative consequences for the insect population in terms of environmental adaption. In order to provide sufficient information regarding this factor, bottleneck studies need to be conducted which cover the genetics of a population as well as the fitness while selecting for resistance in a specific population.

In Chapter 5, negative trade-offs were also shown to be present in strain Sudlon and strain Japan when compared to the susceptible reference strain BCS-S. The fitness costs apparent in strain Japan at 20 °C and 30 °C might be related to resistance and stress caused by low or high temperatures. Significant differences in the fitness between strain Sudlon and strain BCS-S present at all three temperatures cannot be directly linked to resistance or its pleiotropic effects as other factors could play a role in attributing the differences in the genetic background of the two strains. Furthermore, differences in ecotype can lead to variation in development as insects show a regional adaptation attributed to acclimatization and thermal responses of insects regarding latitude and altitude (Addo-Bediako *et al.* 2000, Kimura 2004, Budhi Ram *et al.* 2017). In *Cydia pomonella* high fitness costs, such as low female fecundity, longer larval developmental times and lower body mass, were observed in two resistant lines when comparing them to a susceptible strain (Boivin *et al.* 2001). The authors could not directly link the negative trade-offs to resistance as the strains were not derived from the same line. In other studies it was shown that the fecundity in *C. pomonella* was not affected by novaluron, regardless of whether males or females were exposed (Gökçe *et al.* 2009, Kim *et al.* 2011). Furthermore, negative trade-offs can be condition dependent and become apparent in the most stressful environment (Janmaat and Myers 2005). Temperature in general plays an important role in the fitness of insects as extreme temperatures ranging from 4 to 6 °C or from 34 to 40 °C impair development (Liu *et al.* 2002). It was shown in this study that high temperature, i.e. 30 °C, leads to a reduction of life span, fertility, development time and causes a higher mortality in all four strains (Chapter 5).

In addition, two mutations, L1014F (*kdr*) and T929I, were identified in the voltage-gated sodium channel of strain Sudlon which confer pyrethroid (deltamethrin) resistance (data not shown). It is not clear if these mutations play a role in the observed fitness costs in strain Sudlon, although no fitness costs have been reported so far concerning these mutations in DBM. In summary, fitness costs were present in the highly diamide and BPU resistant strain Sudlon-Tfm when comparing it to its parental strain Sudlon. In the absence of the insecticide, the reduced population growth examined in Sudlon-Tfm could have a greater impact on the overall population size and could result in a population replacement in the field by another population which shows a higher fitness. Thus, benzoylurea selection pressure on a diamide-resistant DBM strain leads to significant reduction of the overall fitness and population growth parameters under applied conditions.

6.3 Resistance Management

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The development of field resistance depends on several factors including the genetic variability already present in a population of pests treated by consecutive applications with the same mode of action, thus facilitating the survival and reproduction of genotypes with a heritable ability to resist such applications at manufacturer recommended label rates. However, steadily increasing but still low levels of resistance are often less obvious under field conditions in terms of initial efficacy, but can result in an incremental reduction of residual activity due to the capacity of selected genotypes to resist declining quantities of active substance which would still provide reasonable control of completely susceptible individuals. In order to prevent or – realistically spoken – delay such a process, resistance management strategies need to be implemented in order to sustain the efficacy of a mode of action or chemical class of insecticide. The introduction of diamide insecticides into global markets was accompanied by communication and educational activities mainly driven by an IRAC International Diamide Working Group as well as more than 20 different IRAC Diamide Country Groups, tying together knowledge including baseline studies on high-risk pests and suitable (regional) IRM strategies in a diverse range of cropping systems (Teixeira and Andaloro 2013). The main objectives of the established regional IRAC Country Teams were (a) the identification and prioritisation of high resistance risk pests and cropping systems; (b) the adaptation of the global IRM guidelines into appropriate regional resistance management strategies; (c) the development of communication strategies particularly facilitating product labelling (IRAC Group 28 insecticides), advertising and education; (d) the communication of IRM recommendations, rotation strategies and optimal number of applications per cropping cycle by a so-called window approach (Fig. 6.1); (e) the development of an extensive education and knowledge transfer programme to train influencers and growers utilising local industry and IRM experts; and, last but by no means least, (f) the implementation of IRM strategies through education and training programmes, both on a global and regional scales (Teixeira and Andaloro 2013, refer also to www.irac-online.org for continuous updates on general IRM strategies, including diamides). A key point of established IRM strategies is the rotation of diamide insecticides between pest generations with other modes of action and to limit the number of applications throughout the cropping cycle by an IRM

window approach (Fig. 6.1). In addition diamides exhibit some favourable application characteristics, such as low effects on populations of most beneficial insects, known to facilitate IRM within integrated pest management programmes. As diamides are distinct from all other chemical classes of insecticides (Sparks and Nauen 2015), they can principally be rotated with all those classes in IRM strategies. Currently, there is no metabolic detoxification mechanism described in any diamide-targeted pest conferring field-relevant cross-resistance to other Lepidoptera-active insecticides, rendering them highly valuable tools for both combining and alternating insecticide modes of action. The predominance and global spread of a target-site-based resistance mechanism in diamondback moth (Steinbach *et al.* 2015) – though recessive – should serve as a warning that resistance development may also easily extend to other pests if these are continuously selected by the treatment of consecutive generations, such as what recently happened for *T. absoluta* in southern Europe (Roditakis *et al.* 2015). However, cases of significant resistance to diamides under applied field conditions are so far regionally restricted to a few Lepidoptera species (see introduction, Table 1.3), with the notable exception of *P. xylostella* (Troczka *et al.* 2012, Wang and Wu 2012, Gong *et al.* 2014, Ribeiro *et al.* 2014, Steinbach *et al.* 2015).

Additionally, BPU should be considered and implemented in RM strategies for controlling lepidopteran pests including diamide resistant DBM populations.
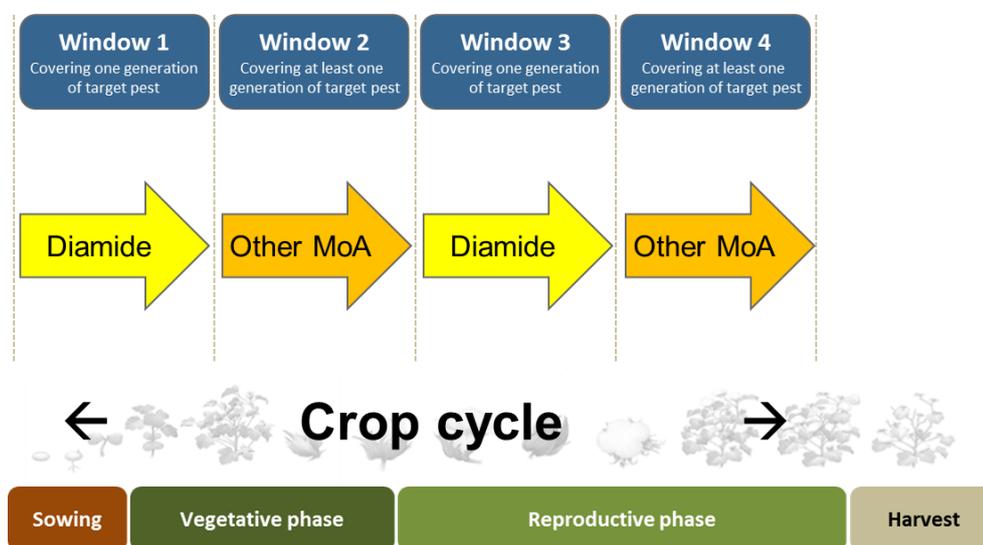
Figure 6.1: Recommended insecticide mode of action rotation practice for resistance management by an application window approach to avoid exposure of consecutive pest generations to the same mode of action such as diamides (IRAC MoA group 28; www.irac-online.org).

6.4 Future work perspectives

Insecticide resistance is rapidly increasing and occurs on a global scale in many pest species.

The genetic changes, however, which confer insecticide resistance, are still not fully understood especially the origins of resistant alleles. The current study focused on the mechanisms underlying diamide as well as BPU resistance and it contributed as a whole to the understanding of the development of resistance. New methods, such as the CRISPR/Cas9 system, were used in order to unravel the mode of action of insect growth regulators. Furthermore, this study provided suggestions for an improvement on pest control measures regarding RM strategies and helps to implement further studies to investigate resistance mechanisms. Profound knowledge of the strategies pests can utilise to overcome insecticide toxicity provided by research activities can in turn support the design and the development of new chemical compounds. Thus, closer cooperation between public/private research and agriculture would be beneficial.

In Chapter 2 and 3 the mechanisms underlying diamide resistance including the genetics and distribution of resistance in diamondback moth and *T. absoluta* were investigated. Target-site mutation G4946E/V and I4790M/T were identified and functional evidence was provided on the implications of these mutations on diamide binding. However, the mutations I4790M and G4946V could not be investigated on a functional level individually as they occurred in combination with the mutation G4946E/V and I4790M, respectively in *T. absoluta*. Therefore, it would be of interest to study the implications of the latter named mutations including E1338D and Q4594L on diamide binding on an individual scale. Unravelling the role of the amino acid at position 4790 in the insect RyR would be of utmost interest in terms of selectivity issues between phthalic and anthranilic acid diamides. Furthermore, it would be interesting to see if mutation G4946V provides the same level of resistance as the glutamic acid residue at the same position. Identifying the role of the single amino acids at the proposed locations in the RyR would help to determine the exact binding region of diamides to the insect RyR.

Using molecular techniques such as cloning and site-directed mutagenesis, mutations could be specifically introduced into the RyR of DBM or *T. absoluta*. Subsequently the altered receptor could be recombinantly expressed in insect cells and cells could be harvested for further studies including and specific saturable binding of selected radioligands. Another approach would be utilizing the CRISPR/Cas9 or alternative gene editing approaches, to introduce the mutations in the orthologous RyR gene in *D. melanogaster*. Thereby, metabolic factors or pleiotropy which might contribute to the resistance could be eliminated as CRISPR/Cas9 provides control over genetic background.

Knowledge of the functional group, its MoA and exact binding site of insecticides could open a new strategy for rationally engineering insecticides with a high insecticidal activity that exhibit greater target specificity as well as selectivity.

In Chapter 5, fitness costs were shown to be involved in strain Sudlon, strain Sudlon-Tfm and strain Japan in comparison to the susceptible strain BCS-S. In order to discern whether these negative trade-offs are caused by resistance and its pleotropic effects, a more rigorous genetic

protocol with repeated introductions of the resistant gene into the same genetic background would be necessary. Furthermore, fitness costs were involved in BPU resistance when comparing strain Sudlon-Tfm with its parental strain Sudlon. It was shown that these costs were not attributed to the target-site mutation identified in CHS-1 in Sudlon-Tfm. Thus, detrimental genes which might play a role could be identified by using next-generation sequencing which has already increased the rate of discovery of resistance genes. Combining this technique with quantitative PCR, gene expression studies could give further information on multigenic resistance factors such as metabolic enzymes that caused the observed fitness costs in Sudlon-Tfm. Next-generation-sequencing also provides a tool for investigating the interactions of different resistance mechanism and uncovering yet undiscovered resistance traits.

Additionally, monitoring needs to be implemented more frequently using bioassays in combination with molecular techniques in order to provide an overview of the pest resistance status of and to support IPM strategies. Furthermore, this is extremely useful and can aid in creating statistical models which could suggest how soon a pest is likely to develop resistance.

6.5 Conclusion

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Diamide insecticides

Diamide insecticides show a remarkable overall activity against lepidopteran pest species, and after 10 years on the market, this chemical class gained blockbuster status economically and considering its global impact in many agricultural and horticultural cropping systems. However, despite their widespread use, diamide resistance development compromising field efficacy is yet restricted to a few, mostly regional cases, except for diamondback moth. Investigations into the molecular mechanisms of diamide resistance in this pest revealed RyR target-site mutations with strong functional implications for diamide binding. This also facilitated fundamental research on the genetics of diamide resistance and associated fitness costs. However, the evolution of target-site resistance is definitely an unpleasant event from an applied perspective, but it also offers opportunities to extend our knowledge on the biochemistry of insect RyRs as

insecticide targets, e.g. by contributing to the understanding of diamide selectivity (insects vs. mammals) and by mapping the elusive diamide binding site, possibly allowing the design of novel ligands overcoming target-site resistance. The fairly rapid evolution of this target-site resistance mechanism in diamondback moth, due to high treatment frequency in tropical conditions, suggests that other pests with a lower number of generations per year and thus less frequently treated are likely to follow soon, if no appropriate IRM strategies as outlined above are implemented, helping to conserve diamide insecticides as a valuable chemical tool for sustainable agriculture.

Benzoylphenyl urea

The successful combination of applied resistance research, genetic studies and genome-editing has led to the discovery of the molecular target site of benzoyl urea insecticides and thus shows the importance of modern research in combating pest resistance. Unravelling the MoA of BPUs was a major milestone in insecticide resistance research as this mechanism had remained elusive for over 40 years. Based on the knowledge that chitin synthesis inhibitors directly interfere with chitin synthase 1, IPM strategies can be better implemented and used to delay the development of resistance. Thus, BPUs possess the valuable property of being non-neurotoxic and bind to a target site that is absent in vertebrates.

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Appendix A

Chapter 3
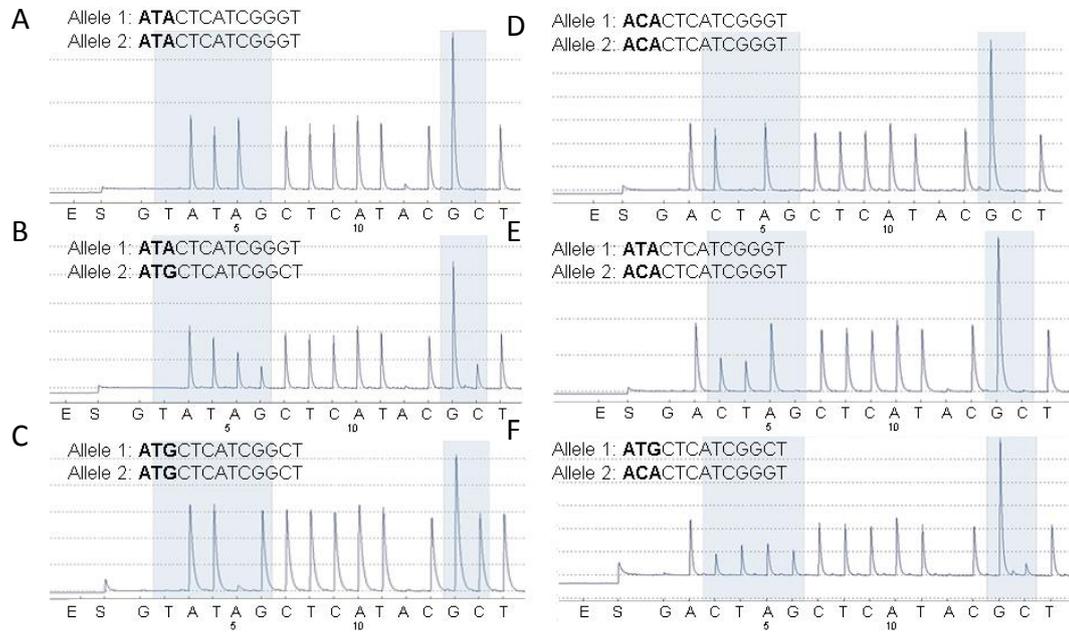
Figure S1. SNP pyrosequencing assay results for RyR mutation I4790M (A-C) and I4790T (D-F) in *T. absoluta*. (A) Homozygous ATA, genotype SS, (B) Heterozygous ATA/ATG, genotype RS, (C) Homozygous ATG, genotype RR. (D) Homozygous ACA, (E) Heterozygous ATA/ACA, genotype RS, (E) Heterozygous ATG/ACA, (D and F) insects have shown a resistant phenotype, genotype presumably RR.
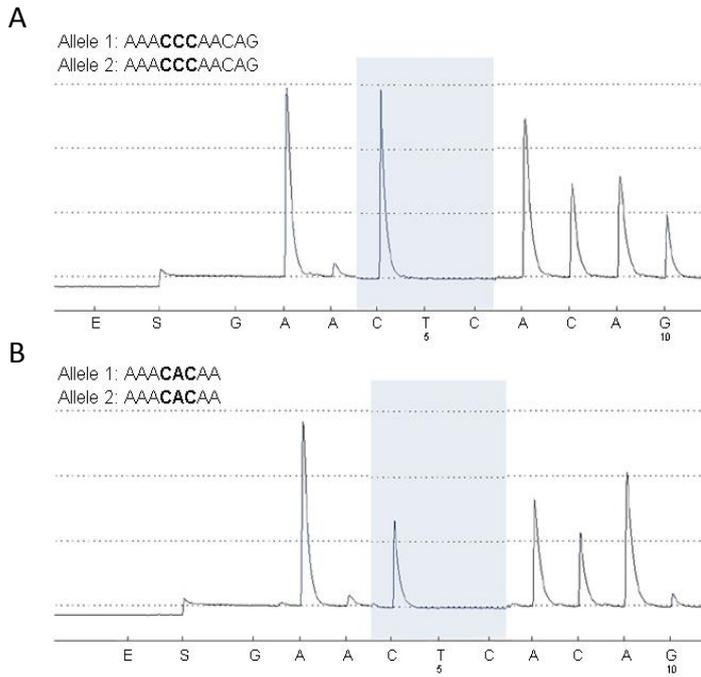
Figure S2. SNP pyrosequencing assay results for RyR mutation G4946V in *T. absoluta*. (A) Homozygous CCC, genotype SS, (B) Homozygous CAC, genotype RR.

Figure S3. Genotyping by pyrosequencing failed with the Brazilian field strains BR-GML1 and BR-PSQ due to polymorphisms in the *RyR* nucleotide sequence covering the regions chosen for primer design and based on a *RyR* sequence obtained from susceptible reference strain (GenBank no. KX519762).

Table S1. List of primers used for amplification of *Tuta absoluta* ryanodine receptor

Primer	Sequence
Ta. F1-F	AGAAATAGTTCATATAGACGAGGAC
Ta. F1-R	ATCATCATAAAGCCGCGCGTCCACC
Ta. F2 F	CAGTGGGAGAAGCCGCAGATCAAGG
Ta. F2-R	GCGGGACCTTGAGGTGGTAGTACC
Ta. F3 F	CAACATTGATATTCAGATGGGCATGC
Ta. F3-R	CGTGCGTCATTTCAAAGATGGCG
Ta. F4 F	TGTGGCAGAAATATTCAACATCTGG
Ta. F4-R	CTGCGCCTTCCAACCCCACACC
Ta. F5 F	GCGTCTCTGTTCTGTAAGCTGGCG
Ta. F5 R	CTTGTCTCGCTTCTTGTCGCGG
Ta. F6 F	TGGTTGCGCTCTACCACCCGACTAC
Ta. F6 R	ATGGTATGCCCCAAATCATCAGC
Ta. F7-F	GACGCCAACAAAATGAGCGAGAGG
Ta. F7-R	CTCGATTTTCCTGGACGCCCAAGC
Ta. F8-F	GAAACTAAGAAGGGTGCATTTAGC
Ta. F8-R	CGTTCTTCCCCAGAGCTATAACTGC
Ta. F9-F	ACGCCTTGTGTCAACACGTGGACC
Ta. F9-R	ACCGCGTCGTATTGCCGGACC
Ta. F10-F	CGTGGCTTCCGAAGAAAAGAAGG
Ta. F10-R	GGCCCAAAGGTATCACGTATTCG
Ta. F11-F	AGCTGACTTCAGGACGTACAGAGC
Ta. F11-R	AGTTGTGGCGATGGAACTGAGAGG
Ta. F12-F	TCGACGGAGCGTATCTCTGGTCC
Ta. F12-R	AGACTCCGAATTACCGCTGTACAC
Ta. F13-F	TCGGCAAAGTGGAAGAGAAACAAGC
Ta. F13-R	AGTTTACTAGAGCAGCTGATAACCG
Ta. F14-F	TGTGCGCGGGAATGGTGCTGGCG
Ta. F14-R	GGTTCTCTTGCAGGGTTTCTAGAC

Table S2. List of primers used for pyrosequencing and Sanger sequencing

Primer	Sequence
Ta_I4790-F	5'-AACATAGCGGCCGCGCTG-3'
Ta_I4790-R-btn	5'-[btn]GCGGGACCTTGAGGTGGTAG-3'
Ta_I4790-Seq-F	5'-TTCCATCGYCTCACTCGCC-3'
Ta_G4946-F-btn	5'-[btn]CGTTCCTCTATTCCCTGTGGT-3'
Ta_G4946-R	5'-CTTCTTCCGCAAGTTCTACGT-3'
Ta_G4946-Seq-R	5'-ATSGTYCTCAACGTCTT-3'
Ta_SangerSeq-F	5'-CATAGACTGGCGBTACCAAGTGT-3'
Ta_SangerSeq-R	5'-TGMACGTARAACTTGCGGAAG-3'

Appendix B

Chapter 4

B1 Material and methods

B1.1 Genome Modification Strategy.

Genomic region sequencing in nos.Cas9 flies.

We used three primer pairs (CHS11F/CHS11R, CHS12F/CHS12R, and CHS20F/CHS20R; Table S2) to generate three overlapping DNA fragments encompassing genomic region 3R: 5380505:5383684 (numbering according to BDGP6 genome assembly) by PCR amplification (see main text), using DNA from y^1 M{nos-Cas9.P}ZH-2A w* flies. This region was sequenced to identify possible variations from the published genome sequence (none detected).

Generation of gRNA plasmids.

We used gRNA vector pU6-*Bbs*I-chiRNA (Gratz *et al.* 2013), after digestion with *Bbs*I, and we ligated specific double-stranded DNA oligos generated by annealing of single-stranded DNA oligos 444F/444R and 658F/658R (Table S2) to generate RNA expressing plasmids gRNA444 and gRNA658, respectively, targeting the relevant genomic regions (Fig. S5).

Donor plasmids for HDR.

We constructed de novo (Genscript) two donor plasmids for HDR encompassing genomic region 3R:5380538:5383542 but with certain modifications compared with the wild-type genomic sequence (Fig. S5): for the I1056F mutation (equivalent to position 1042 in *P. xylostella* and 1017 in *T. utrticae*), a C→T synonymous transition that abolishes an NcoI restriction site in the donor sequence and an A→T transversion that generates the I1056F mutation (codon alteration ATC→TTC) and abolishes a *Fok*I restriction site in the donor sequence (Fig. S5). For the I1056M mutation, apart from the C→T synonymous transition abolishing the NcoI site and the C→G transversion that generates the I1056M mutation (codon alteration ATC→ATG), we added six more synonymous changes, three on each of the gRNA CRISPR targets (Figs. S5), to avoid cleavage of the donor plasmid by the CRISPR/Cas9 procedure used to target the genome.

***Drosophila* embryo injection and G_1 screening.**

Embryos were injected with a plasmid mixture containing 75 ng/μL of each gRNA plasmid 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 by isolating DNA from sets of ~30 individuals per vial (mostly pupae, but also adults and third instar larvae, depending on availability), digesting ~2 μg of total DNA with NcoI (to preferentially digest wild-type alleles but not the modified alleles that contain the donor sequence where NcoI is absent) and using ~30 ng of the digested DNA as template for amplification either with "mutant-specific" primer pairs (ETXSF/ETXSM yielding a 247-bp product for I1056F or ETXSF/PxM yielding a 249-bp product for I1056M) or a "generic" (ETXSF/ETXSR) primer pair (Table S2) yielding a 395-bp product (Fig. S6). In case the generic primer pair was used, the product was further digested with NcoI and/or *Fok*I for allele evaluation. For individual flies, screening was performed as described above but with template DNA derived from individual fly DNA preps (2–5 ng per reaction).

B1.2 Life Table Parameters.

P. xylostella life tables.

Strains Sudlon and Sudlon-Tfm were placed in plastic cages in a climate chamber and kept under standard conditions at 25 ± 1 °C, relative humidity $50 \pm 10\%$, and LD 16:8 for one generation to adapt to the conditions. To investigate postembryonic developmental stages, 50 adults of each strain were transferred to a cage, and females were allowed to lay eggs for 6 h; afterward the cabbage plants were removed, and egg development was monitored. Fifty newly hatched first instar larvae were transferred to a six-well plate containing a wetted filter paper and a cabbage leaf disk (2-cm diameter). Development was recorded every 6 h until pupation. Data were analyzed for significant differences between strains by Student's *t* test.

Drosophila life tables.

To determine the developmental time from egg to pupa/adult, cages with 20 adult females and 20 adult males were covered with cherry-agar plates layered with yeast. The flies were left to adapt for several hours, and after plate replacement, they were left to lay eggs for ~5 h. Fifty eggs were transferred in small vials with fly diet (five replicates for each line) and left to grow in standard conditions (25 ± 1 °C, relative humidity $70 \pm 10\%$, LD 12:12). Pupation and adult eclosion timing and number of pupae/adults were monitored. For determination of fecundity, 20 crosses (one virgin female × one male) per line were set into vials covered with a cherry-agar plate. The number of eggs was counted, and the plate was replaced daily. Data were analyzed for significant differences between strains by Student's *t* test.

B2 Results

Insect genomes typically contain two genes for CHS with different developmental roles: CHS1 is only expressed at molting, whereas CHS2 is continuously expressed in midgut. To verify which of the two *Drosophila* candidate genes (*kkv* or CHS2) is more related to the *Plutella* CHS, the percentage of sequence similarity was calculated and it was found that the *P. xylostella* CHS1 is significantly more related to *kkv* (sequence identity 68.3%) than to *Drosophila* CHS-2 (37.6%). The *T. urticae* CHS1 shares 48.6% identity with *kkv* and 34% with *Drosophila* CHS2. Furthermore, a phylogenetic analysis of several arthropod CHSs (Fig. S4) clearly indicates that *kkv* is grouped together with *P. xylostella* and other insect CHS1 proteins in a clade with strong support, whereas CHS2 belongs to a different clade. Thus, *kkv* was considered the appropriate target for genome modification.

Table S1. Log-dose probit-mortality data for triflumuron tested against instar larvae of diamondback moth strains BCS-S, Sudlon, and Sudlon-Tfm as well as their respective reciprocal crosses (F1).

Strain	n	LC$_{50}$, ppm	95% CL[*]	SLOPE	RR[†]	D[‡]
BCS-S	630	6.1	4.6–8.2	0.95		
Sudlon	420	18	11–30	0.89	3	
Sudlon-Tfm	180	>1,000			>164	
Reciprocal crosses						
F1[§]	210	12	9.2–16	0.81	2	−0.73
F1[¶]	240	23	17–31	0.78	1	−0.88

[*] 95% confidence limits.
[†] Resistance ratio.
[‡] Degree of dominance.
[§] BCS-S × Sud-Tfm.
[¶] Sudlon × Sud-Tfm.
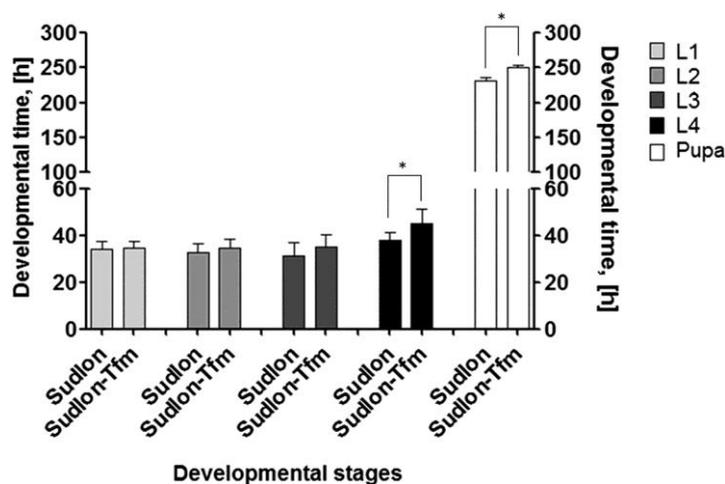
Fig. S2. Comparison of the postembryonic developmental time (±SD) of strains Sudlon and Sudlon-Tfm. Sudlon-Tfm shows a significant longer larval (L4) and pupal development ($P < 0.0005$, $R^2 = 0.3$, $n = 50$).
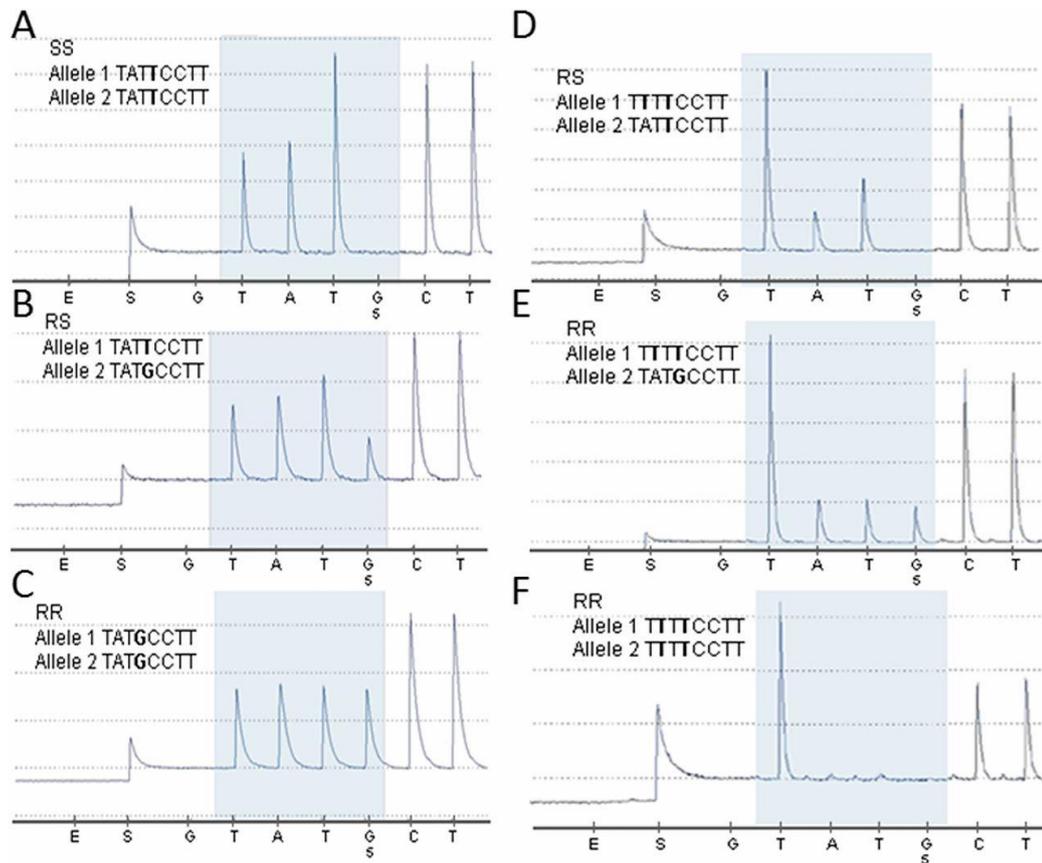
Fig. S3. SNP pyrosequencing assay results for the CHS I1042M mutation (ATT/ATG, printed in bold letters) in amplified cDNA and gDNA fragments of *P. xylostella*. (*A*) Homozygous ATT, genotype SS. (*B*) Heterozygous ATT/ATG, genotype RS. (*C*) Homozygous ATG, genotype RR. (*D*) Heterozygous TTT/ATT, genotype RS. (*E*) Heterozygous TTT/ATG, genotype RR. (*F*) Homozygous TTT, genotype RR.
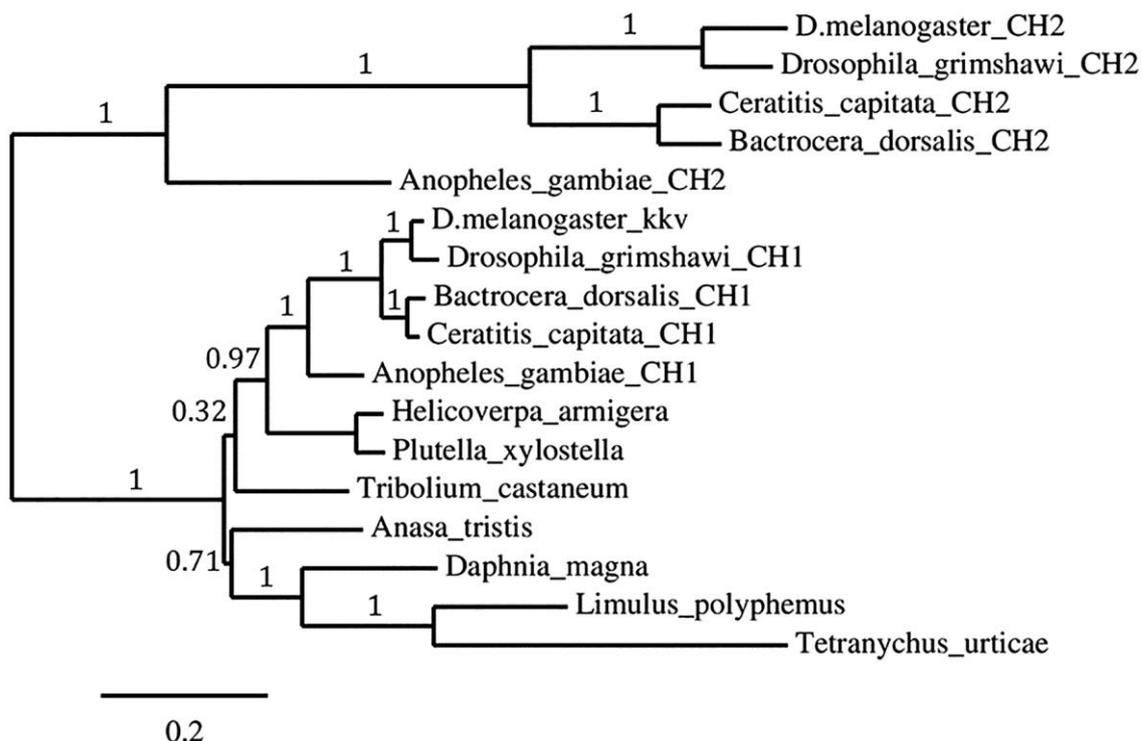
Fig. S4. Phylogenetic analysis of several arthropod CHS protein sequences. Dipteran CHSs: *A. gambiae* (accession no. CH1: XP_321336.5, CH2: XP_321951.2), *Bactrocera dorsalis* (CHS1: XP_011203784.1, CHS2: AGC38392.1), *Ceratitis capitata* (CHS1: XP_012157009.1, CHS2: XP_012161954.1), *Drosophila grimshawi* (CHS1: XP_001994028.1, CHS2: XP_001985562.1), *D. melanogaster* (*kkv*: NP_524233.1, CHS2: NP_524209.3). Other insect CHSs: *P. xylostella* (Lepidoptera, BAF47974.1), *Helicoverpa armigera* (Lepidoptera, AKJ54482.1), *Tribolium castaneum* (Coleoptera, NP_001034491.1), *Anasa tristis* (Hemiptera, AFM38193.1). Other arthropod CHSs include spider mite *T. urticae* (XP_015781017.1), horseshoe crab *Limulus polyphemus* (XP_013790798.1), and crustacean *Daphnia magna* (KZS08010.1). Phylogenetic analysis was performed as described in Dereeper *et al.* (2008) using the one-click mode (www.phylogeny.fr/index.cgi). Numbers on branches indicate the approximate likelihood ratio test confidence index.

```
1     ACCAGCGGCGCAGATGGGTGCCCTCGACCATAGCCAACATCATGGATCTTCTGGCGGATGCGAAGCGCACGATCAAGATC   80
      N  Q  R  R  W  V  P  S  T  I  A  N  I  M  D  L  L  A  D  A  K  R  T  I  K  I

81    AATGACAACATTTCGCTTCTGTACATCTTCTACCAGATGATGTTGATGGGCGGCACCATCCTGGGACCCGGAACCATTTT   160
       N  D  N  I  S  L  L  Y  I  F  Y  Q  M  M  L  M  G  G  T  I  L  G  P  G  T  I  F

161   CCTTATGTTGGTGGGTGCATTCGTGGCAGCCTTTCGCATTGACAACTGGACCTCCTTCCACTACAACATCGTGCCGATCC   240
       L  M  L  V  G  A  F  V  A  A  F  R  I  D  N  W  T  S  F  H  Y  N  I  V  P  I

                                               ETXSF
241   TGGCCTTCATGTTTATCTGCTTCACCTGTAAGTCGAACATCCAGTTGTTCGTGGCCCAAGTCCTTTCGACGGCATATGCC   320
       L  A  F  M  F  I  C  F  T  C  K  S  N  I  Q  L  F  V  A  Q  V  L  S  T  A  Y  A

321   CTGATCATGATGGCGGTGATTGTGGGTACGGCCTTGCAGTTGGGAGAGGACGGAATAGGCTCCCCTTCGGCCATTTTCCT   400
       L  I  M  M  A  V  I  V  G  T  A  L  Q  L  G  E  D  G  I  G  S  P  S  A  I  F  L

                                                      PAM        gRNA444
                                                            G           T  C
401   GATATCCATGGTGGGCTCATTCTTCATAGCCGCATGTTTGCATCCACAAGAGTTCTGGTGCATTACATGCGGCCTAATCT   480
       I  S  M  V  G  S  F  F  I  A  A  C  L  H  P  Q  E  F  W  C  I  T  C  G  L  I

                     (ATC→TTC, I1056F,
                      ATC→ATG, I1056M)

                     I  G     ETXSM
481   ATTTGCTGTCCGACCCCGTCCATGTACCTGCTGCTCATCTTGTACTCTATCATCAACCTAAACGTCGTCTCCTGGGGCACC   560
       Y  L  L  S  T  P  S  M  Y  L  L  L  I  L  Y  S  I  I  N  L  N  V  V  S  W  G  T
                                PxM

561   CGCGAGGTGGTGGCTAAGAAGACCAAGAAAGAACTGGAGGCGGAGAAGAAGGCCGCCGAGGAGGCCAAAAAGAGGGTGAA   640
       R  E  V  V  A  K  K  T  K  K  E  L  E  A  E  K  K  A  A  E  E  A  K  K  R  V  K

                          gRNA658              PAM
                           G     C       T
      ETXSR
641   GCAGAAGAGCATGCTGAGCTTCCTTCAGAGTGGAATCGGGGACAATGGCGACGAGGAGGGCTCCGTGGAGTTCTCTCTCG   720
       Q  K  S  M  L  S  F  L  Q  S  G  I  G  D  N  G  D  E  E  G  S  V  E  F  S  L

721   CTGGACTCTTCCGCTGCATATTCTGCACCCACGGAAAAACATCCGACGAGAAGCAGCAGCTGACCTCCATCGCCGAATCG   800
       A  G  L  F  R  C  I  F  C  T  H  G  K  T  S  D  E  K  Q  Q  L  T  S  I  A  E  S
```

Fig. S5. Nucleotide and deduced amino acid sequence of an 800-bp fragment of *kkv* exon 6 (corresponding to 3R:5381406:5382206 at the BDGP6 genome assembly), flanking position 1056 of the *D. melanogaster* amino acid sequence (*I*, shown in black background), equivalent to 1042 in *Plutela xylostella* and 1017 in *T. urticae*. Light gray areas indicate the CRISPR/Cas9 targets selected (gRNA444, gRNA658), whereas dark gray areas indicate the corresponding PAM (–*NGG*) triplets. Vertical arrows denote break points for CRISPR/Cas9-induced double stranded breaks. Ovals mark differences between target (wild-type) and donor (genome modified) sequences (red for I1056M and blue for I1056F). A C→T synonymous transition common for both designs at position 407 abolishes an NcoI cleavage site (CCATGG, underlined); an A→T transversion at position 492 generates a codon alteration (ATC→TTC) that results in the I1056F mutation, whereas a C→G transversion at position 494 generates a different codon alteration (ATC→ATG) that results in the I1056M mutation. Six extra synonymous mutations present at the CRISPR targets in the I1056M design are shown in red letters. Horizontal arrows indicate the relative positions of the primers used for diagnostic screening [ETXSF, ETXSR, ETXSM (blue, specific for I1056F), and PxM (red, specific for I1056M); see Table S2].
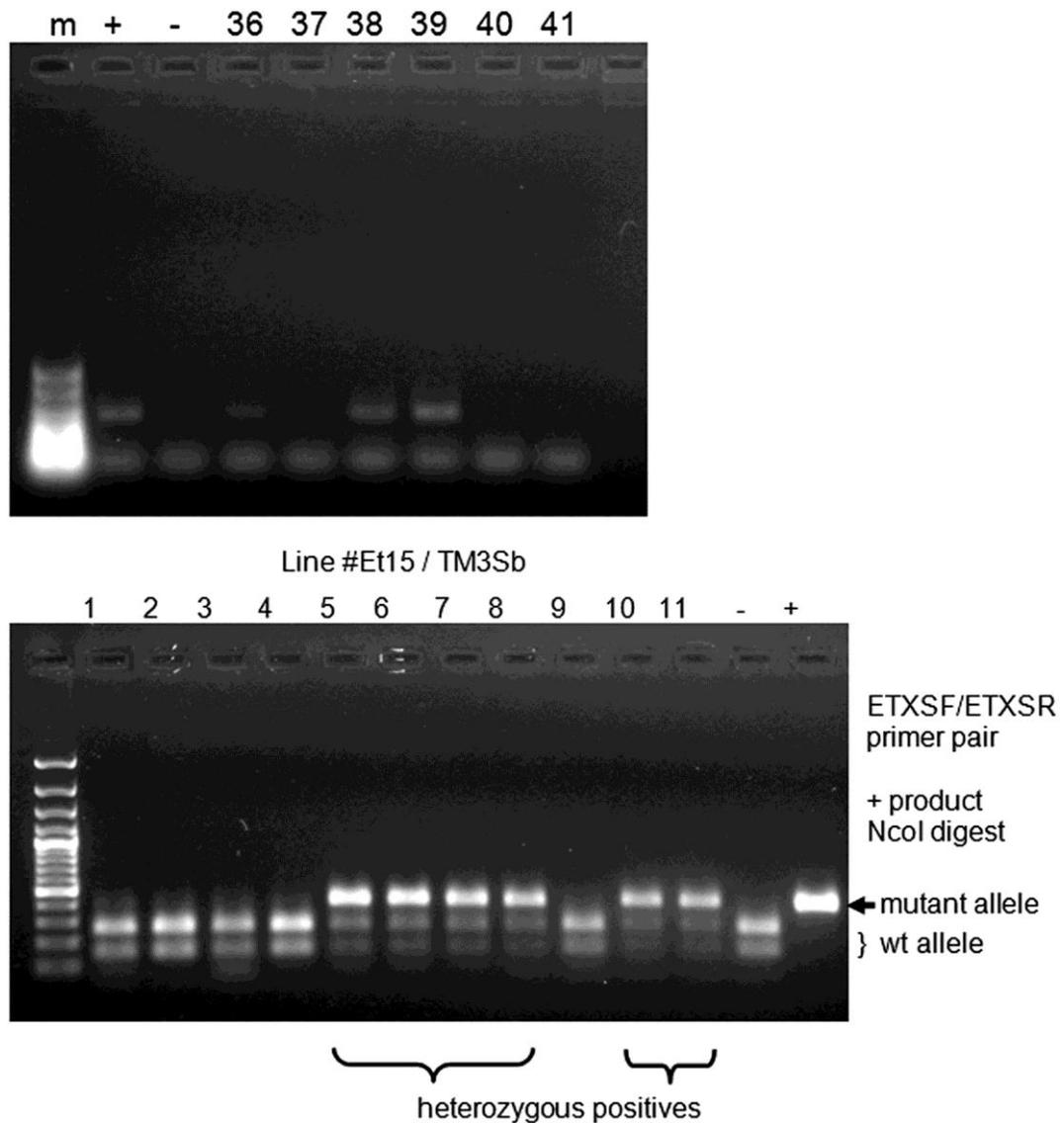
Fig. S6. Screening for genome-modified flies. (*Top*) PCR screening after digestion with NcoI of template DNA from pools of G_1 flies derived from different G_0 (injected) individuals using a specific primer pair (ETXS/PxM) for I1056M mutation [–, nos.Cas9 DNA (negative control); +, donor plasmid template (positive control)]. (*Bottom*) Screening of G_2 flies from line Et15 crossed to balancer TM3*Sb*, PCR amplification with a generic primer pair (ETXSF/ETXSR), and digestion of 395-bp product with NcoI. The modified allele remains uncut, whereas the wild-type allele is cut in two smaller bands; positives are heterozygous at this stage.

Eclosion

Daily egg laying
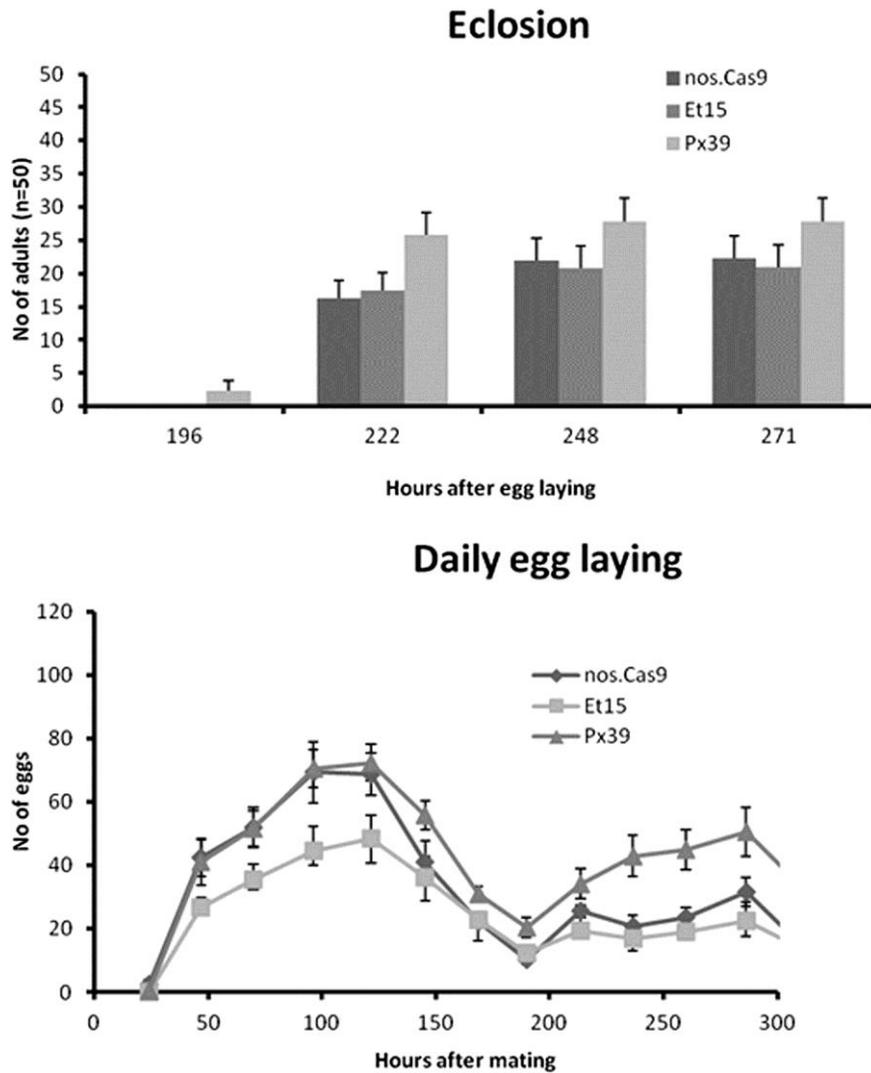
Fig. S7. (*Top*) Comparison of developmental timing and number of eclosed adults out of 50 original eggs among different *Drosophila* lines used in the bioassays of this study. No significant difference was found between Px39 (I1042M) or Et15 (I1017F) versus nos.Cas9 wild-type controls ($P = 0.151$ and $P = 0.4$, respectively). (*Bottom*) Comparison of average daily fecundity among lines ($n = 8$ for Et15 and nos.Cas9, $n = 7$ for Px39). No evidence for reduced fecundity is found for line Px39 (I1042M). Error bars represent SEM.

Appendix C

Chapter 5

Table S1. Life table parameters of the insecticide susceptible diamondback moth strain BCS-S at different temperatures.

			20°C BCS-S						
Stage x	**Age (days)**	**ax**	**lx**	**dx**	**qx**	**Lx**	**Tx**	**ex**	**sx**
Egg	0	50	1.00	0.00	0.04	1.00	6.20	6.20	0.96
Instar I	3.31	50	1.00	0.08	0.08	0.96	5.20	5.20	0.92
Instar II	4.82	46	0.92	0.02	0.02	0.91	4.24	4.61	0.98
Instar III	6.74	45	0.90	0.04	0.04	0.88	3.33	3.70	0.96
Instar IV	8.64	43	0.86	0.04	0.05	0.84	2.45	2.85	0.95
Prepupa	11.06	41	0.82	0.02	0.02	0.81	1.61	1.96	0.98
Pupa	11.84	40	0.80	0.00	0.00	0.80	0.80	1.00	1.00
Adult	16.14	40	0.80						
			25°C BCS-S						
Stage x	**Age (days)**	**ax**	**lx**	**dx**	**qx**	**Lx**	**Tx**	**ex**	**sx**
Egg	0	50	1.00	0.00	0.10	1.00	6.27	6.27	0.90
Instar I	2.81	50	1.00	0.06	0.06	0.97	5.27	5.27	0.94
Instar II	4.2	47	0.94	0.04	0.04	0.92	4.30	4.57	0.96
Instar III	5.47	45	0.90	0.02	0.02	0.89	3.38	3.76	0.98
Instar IV	6.62	44	0.88	0.04	0.05	0.86	3.40	3.86	0.95
Prepupa	8.07	42	0.84	0.02	0.02	0.83	1.63	1.94	0.98
Pupa	8.7	41	0.82	0.04	0.05	0.80	0.80	0.98	0.95
Adult	12.23	39	0.78						
			30 °C BCS-S						
Stage x	**Age (days)**	**ax**	**lx**	**dx**	**qx**	**Lx**	**Tx**	**ex**	**sx**
Egg	0	100	1.00	0.00	0.16	1.00	4.85	4.85	0.84
Instar I	2.63	100	1.00	0.14	0.14	0.93	3.85	3.85	0.86
Instar II	3.63	86	0.86	0.14	0.16	0.79	2.92	3.40	0.84
Instar III	4.63	72	0.72	0.10	0.14	0.67	2.13	2.96	0.86
Instar IV	5.58	62	0.62	0.06	0.10	0.59	2.43	3.92	0.90
Prepupa	6.62	56	0.56	0.05	0.09	0.54	0.87	1.55	0.91
Pupa	7.09	51	0.51	0.35	0.69	0.34	0.34	0.66	0.31
Adult	10.24	16	0.16						

ax   living at the beginning of stage

lx   survival from birth to age x, proportion surviving to each life stage: ax/a0

dx   number of individuals dead during age class X, lx–lx+1

qx   age-specific mortality rate, qx= dx/lx

Lx   Mean number alive at x, (nx+nx+1)/2

Tx   total number of living individuals at age x and beyond; Tx = Lx + (Lx + 1) …+ (Lx + n)

ex   members surviving to a particular age, Tx/lx

sx   age specific survival, lx+1/lx

Table S2. Life table parameters of the insecticide resistant diamondback moth strain Japan at different temperatures.

			20°C Japan						
Stage x	**Age (days)**	**ax**	**lx**	**dx**	**qx**	**Lx**	**Tx**	**ex**	**sx**
Egg	0	50	1.00	0.00	0.08	1.00	6.31	6.31	0.92
Instar I	3.33	50	1.00	0.02	0.02	0.99	5.31	5.31	0.98
Instar II	4.87	49	0.98	0.06	0.06	0.95	4.32	4.41	0.94
Instar III	6.8	46	0.92	0.04	0.04	0.90	3.37	3.66	0.96
Instar IV	8.74	44	0.88	0.04	0.05	0.86	2.47	2.81	0.95
Prepupa	11.24	42	0.84	0.04	0.05	0.82	1.61	1.92	0.95
Pupa	12.03	40	0.80	0.02	0.03	0.79	0.79	0.99	0.98
Adult	16.92	39	0.78						
			25°C Japan						
Stage x	**Age (days)**	**ax**	**lx**	**dx**	**qx**	**Lx**	**Tx**	**ex**	**sx**
Egg	0	50	1.00	0.00	0.06	1.00	6.16	6.16	0.94
Instar I	2.8	50	1.00	0.06	0.06	0.97	5.16	5.16	0.94
Instar II	4.2	47	0.94	0.06	0.06	0.91	4.19	4.46	0.94
Instar III	5.6	44	0.88	0.02	0.02	0.87	3.28	3.73	0.98
Instar IV	6.76	43	0.86	0.04	0.05	0.84	3.36	3.91	0.95
Prepupa	8.12	41	0.82	0.04	0.05	0.80	1.57	1.91	0.95
Pupa	8.71	39	0.78	0.02	0.03	0.77	0.77	0.99	0.97
Adult	12.17	38	0.76						
			30 °C Japan						
Stage x	**Age (days)**	**ax**	**lx**	**dx**	**qx**	**Lx**	**Tx**	**ex**	**sx**
Egg	0	100	1.00	0.00	0.18	1.00	4.94	4.94	0.82
Instar I	2.7	100	1.00	0.13	0.13	0.94	3.94	3.94	0.87
Instar II	3.73	87	0.87	0.13	0.15	0.81	3.00	3.45	0.85
Instar III	4.8	74	0.74	0.09	0.12	0.70	2.20	2.97	0.88
Instar IV	5.875	65	0.65	0.07	0.11	0.62	2.41	3.71	0.89
Prepupa	6.955	58	0.58	0.06	0.10	0.55	0.89	1.53	0.90
Pupa	7.465	52	0.52	0.37	0.71	0.34	0.34	0.64	0.29
Adult	10.685	15	0.15						

ax   living at the beginning of stage

lx   survival from birth to age x, proportion surviving to each life stage: ax/a0

dx   number of individuals dead during age class X, lx–lx+1

qx   age-specific mortality rate, qx= dx/lx

Lx   Mean number alive at x, (nx+nx+1)/2

Tx   total number of living individuals at age x and beyond; Tx = Lx + (Lx + 1) …+ (Lx + n)

ex   members surviving to a particular age, Tx/lx

sx   age specific survival, lx+1/lx

Table S3. Life table parameters of the insecticide resistant diamondback moth strain Sudlon at different temperatures.

Stage x	Age (days)	ax	lx	dx	qx	Lx	Tx	ex	sx
				20°C Sudlon					
Egg	0	50	1.00	0.00	0.06	1.00	6.13	6.13	0.94
Instar I	3.35	50	1.00	0.08	0.08	0.96	5.13	5.13	0.92
Instar II	4.96	46	0.92	0.04	0.04	0.90	4.17	4.53	0.96
Instar III	6.9	44	0.88	0.04	0.05	0.86	3.27	3.72	0.95
Instar IV	8.87	42	0.84	0.02	0.02	0.83	2.41	2.87	0.98
Prepupa	11.42	41	0.82	0.04	0.05	0.80	1.58	1.93	0.95
Pupa	12.19	39	0.78	0.00	0.00	0.78	0.78	1.00	1.00
Adult	17.14	39	0.78						
				25°C Sudlon					
Egg	0	50	1.00	0.00	0.08	1.00	6.07	6.07	0.92
Instar I	**2.87**	50	1.00	0.08	0.08	0.96	5.07	5.07	0.92
Instar II	4.3	46	0.92	0.06	0.07	0.89	4.11	4.47	0.93
Instar III	5.67	43	0.86	0.04	0.05	0.84	3.22	3.74	0.95
Instar IV	6.97	41	0.82	0.02	0.02	0.81	3.28	4.00	0.98
Prepupa	8.56	40	0.80	0.02	0.03	0.79	1.57	1.96	0.98
Pupa	9.2	39	0.78	0.00	0.00	0.78	0.78	1.00	1.00
Adult	12.69	39	0.78						
				30°C Sudlon					
Egg	0	100	1.00	0.00	0.15	1.00	5.10	5.10	0.85
Instar I	2.65	100	1.00	0.13	0.13	0.94	4.10	4.10	0.87
Instar II	3.69	87	0.87	0.09	0.10	0.83	3.16	3.63	0.90
Instar III	4.78	78	0.78	0.09	0.12	0.74	2.34	2.99	0.88
Instar IV	5.83	69	0.69	0.06	0.09	0.66	2.49	3.61	0.91
Prepupa	6.96	63	0.63	0.07	0.11	0.60	0.94	1.49	0.89
Pupa	7.47	56	0.56	0.43	0.77	0.35	0.35	0.62	0.23
Adult	10.87	13	0.13						

ax   living at the beginning of stage
lx   survival from birth to age x, proportion surviving to each life stage: ax/a0
dx   number of individuals dead during age class X, lx–lx+1
qx   age-specific mortality rate, qx= dx/lx
Lx   Mean number alive at x, (nx+nx+1)/2
Tx   total number of living individuals at age x and beyond; Tx = Lx + (Lx + 1) …+ (Lx + n)
ex   members surviving to a particular age, Tx/lx
sx   age specific survival, lx+1/lx

Table S4. Life table parameters of the insecticide resistant diamondback moth strain Sudlon-Tfm at different temperatures.

Stage x	Age (days)	ax	lx	dx	qx	Lx	Tx	ex	sx
20°C Sudlon-Tfm									
Egg	0	50	1.00	0.00	0.06	1.00	6.27	6.27	0.94
Instar I	3.36	50	1.00	0.06	0.06	0.97	5.27	5.27	0.94
Instar II	5.01	47	0.94	0.04	0.04	0.92	4.30	4.57	0.96
Instar III	7.03	45	0.90	0.02	0.02	0.89	3.38	3.76	0.98
Instar IV	9.06	44	0.88	0.02	0.02	0.87	2.49	2.83	0.98
Prepupa	11.67	43	0.86	0.06	0.07	0.83	1.62	1.88	0.93
Pupa	12.5	40	0.80	0.02	0.03	0.79	0.79	0.99	0.98
Adult	17.53	39	0.78						
25°C Sudlon-Tfm									
Stage x	Age (days)	ax	lx	dx	qx	Lx	Tx	ex	sx
Egg	0	50	1.00	0.00	0.04	1.00	6.31	6.31	0.96
Instar I	2.88	50	1.00	0.04	0.04	0.98	5.31	5.31	0.96
Instar II	4.33	48	0.96	0.02	0.02	0.95	4.33	4.51	0.98
Instar III	5.77	47	0.94	0.06	0.06	0.91	3.38	3.60	0.94
Instar IV	7.24	44	0.88	0.04	0.05	0.86	3.39	3.85	0.95
Prepupa	9.12	42	0.84	0.04	0.05	0.82	1.61	1.92	0.95
Pupa	9.78	40	0.80	0.02	0.03	0.79	0.79	0.99	0.98
Adult	13.56	39	0.78						
30°C Sudlon-Tfm									
Stage x	Age (days)	ax	lx	dx	qx	Lx	Tx	ex	sx
Egg	0	100	1.00	0.00	0.15	1.00	5.06	5.06	0.85
Instar I	2.68	100	1.00	0.11	0.11	0.95	4.06	4.06	0.89
Instar II	3.74	89	0.89	0.12	0.13	0.83	3.12	3.50	0.87
Instar III	4.85	77	0.77	0.10	0.13	0.72	2.29	2.97	0.87
Instar IV	5.96	67	0.67	0.06	0.09	0.64	2.52	3.75	0.91
Prepupa	7.12	61	0.61	0.05	0.08	0.59	0.93	1.52	0.92
Pupa	7.66	56	0.56	0.44	0.79	0.34	0.34	0.61	0.21
Adult	11.18	12	0.12						

ax	living at the beginning of stage
lx	survival from birth to age x, proportion surviving to each life stage: ax/a0
dx	number of individuals dead during age class X, lx–lx+1
qx	age-specific mortality rate, qx= dx/lx
Lx	Mean number alive at x, (nx+nx+1)/2
Tx	total number of living individuals at age x and beyond; Tx = Lx + (Lx + 1) …+ (Lx + n)
ex	members surviving to a particular age, Tx/lx
sx	age specific survival, lx+1/lx

Table S5. Fertility life table.

Temperature	Strain	Parameter		
		Mx	mx	lxmx
20 °C	BCS-S	214	97	78
	Japan	172	78	61
	Sudlon	180	90	70
	Sudlon-Tfm	176	88	68
25 °C	BCS-S	223	111	87
	Japan	189	94	72
	Sudlon	186	93	73
	Sudlon-Tfm	172	78	61
30 °C	BCS-S	104	53	8
	Japan	89	45	7
	Sudlon	101	51	7
	Sudlon-Tfm	99	50	6

Mx =   total eggs per female

mx =   number of females produced per female

Lxmx = The mean number of female offspring produced by females

Curriculum Vitae

EDUCATION

11/2013 – 10/2016   **Ph.D. student**

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WORK EXPERIENCE

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15/11/–18/11/2015   Conference Paper at Entomology 2015, Entomological Society
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14/09/–16/09/2015   Conference Paper at Resistance 2015, Rothamsted, UK

03/2015 – to date   Member of the German Scientific Society for Plant Protection
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02/2015 – to date   Member of the Entomological Society of America

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PUBLICATIONS

Steinbach, D., Moritz, G., Nauen, R., 2017. Fitness costs and life table parameters of highly insecticide-resistant strains of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) at different temperatures. Pest Manag. Sci. 73(9): 1789–1797.

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EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Quellen oder Hilfsmittel verwendet habe.

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Ich erkläre weiterhin, dass ich außer meinem Bachelor sowie Master of Science in Biologie an der Martin-Luther-Universität Halle keine akademischen Grade erworben oder zu erwerben versucht habe.

Statement of authorship

I hereby declare on my honor that this thesis has been composed by me and is based on my own work, unless stated otherwise. All references and verbatim extracts have been quoted, and all sources of information have been specifically acknowledged.

This complete work or in a similar form has not been submitted for any other degree.

Furthermore, I declare that I have not acquired or have tried to aquire any other academic degrees than my Bachelor and Master of Science in Biology at the Martin-Luther-University of Halle.
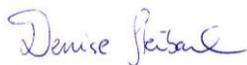
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