Friends with benefits?

– Does gut microbiota of *Spodoptera littoralis* affect insecticide resistance and are there any costs of insecticide resistance development?

Friends with benefits? – Påverkar tarmmikrobiota insekticidresistensen hos Spodoptera littoralis och finns det kostnader för resistensutvecklingen?

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Abstract

The insect gut microbiota has many important functions for insects such as detoxification of host plant toxins. Recently there has been a growing interest on the effect of insect gut microbiota on insecticide resistance development. Insecticide resistance is a growing concern for food security and sustainable agriculture. More knowledge about the relationship between gut microbiota and insecticide resistance development might help to gain more insight into the ecology behind resistance development and to refine pest management strategies.

In this thesis I aimed to understand if gut microbiota can affect insecticide resistance, if there are any costs of resistance development and if gut microbiota can mediate such costs as well as any potential consequences of pesticide exposure on insect life history traits. To answer these research questions, I tested how the gut microbiota of a Cypermethrin-resistant and a susceptible *Spodoptera littoralis* lab strain affected survival of exposition with the insecticide Cypermethrin. The larvae had either been treated with antibiotics (Streptomycin + Ampicillin) prior to the exposition experiments or not, and thus had either a reduced or intact gut microbiota. The larvae that had been treated with antibiotics prior to the insecticide exposition continued to receive antibiotics after exposition as well. Following the exposition experiment I observed life history traits of the insects for the rest of the insect generation and recorded larval growth rate, larval development time, pupation rate, pupal weight, pupal development time, eclosion rate, survival until adulthood and female adult life span. Furthermore, I performed an oviposition experiment to measure female fecundity. The results showed that survival of insecticide exposition was higher for the resistant strain and for larvae with damaged gut microbiota from both the resistant and the susceptible strain. Insecticide resistance did not seem to depend on detoxification through resistant gut bacteria. Insecticide exposition had a negative effect on larval survival but increased larval growth rate, pupal weight, and fecundity. Thus, consequences of insecticide exposure might be long lasting and reach beyond and arise later than the initial survival following exposition. The resistant strain had shorter larval and pupal development time and increased pupation rate, but lower larval growth rate, pupal weight, fecundity, and survival until adulthood compared to the susceptible strain. Thus, resistance development seemed to create fitness costs for resistant insects. Gut microbiota seemed to have a mediating effect on the costs of resistance as well as on the consequences of insecticide exposition.

My results thus indicate that gut microbiota is not contributing to Cypermethrin resistance of *S. littoralis* larvae. Instead, insecticide resistance may increase if pathogenic gut bacteria are removed. My results indicate further that both insecticide exposure, insecticide resistance and gut microbiota presence could have positive or negative effects on *S. littoralis* larvae depending on life stage and whether traits are involved in growth or survival. Implications of these results for pest control and further research are discussed.

Keywords: insecticide resistance development, gut microbiota, gut symbiont-mediated resistance, trade-off effects, fitness costs, symbiosis, survival, life-history traits, fecundity

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1. Introduction

1.1. Insecticides in agriculture

Global food security is under pressure due to population growth, accelerated urbanization, motorization, diet changes such as increasing meat and dairy consumption, and climate change (Popp et al. 2013). The global demand for food, feed and fiber is projected to increase by 70% until 2050 and in addition an increase in the demand of crops grown for energy fuel is expected (FAO 2009). Since arable land is limited and can only be expanded with great loss of biodiversity and vital ecosystem services, productivity will have to be increased on existing farmland *(*FAO 2009; Popp et al. 2013*)*. Without any plant protection measures, the total global yield loss due to agricultural pests would amount to 50-80% for several important staple crops, thereof 9-37% due to animal pests (Oerke 2006). And global yield loss of important staple feed crops due to insect pests might furthermore increase by 10-25% for every degree of global mean surface warming (Deutsch et al. 2018). Due to all of these circumstances, reducing current yield losses is an important challenge (Popp et al. 2013). Increased human population growth during the 20th century has been possible due to industrial agriculture and the use of synthetic fertilizers and pesticides and due to present day increased global demand of food, the use of synthetic pesticides will likely continue in the near future (Carvalho 2017; de O. Gomes et al. 2020). However, excessive use of insecticides and other pesticides can contaminate terrestrial and aquatic ecosystems, impact non-target animals and cause severe human health problems and fatalities (Ansari et al. 2014; Carvalho 2017; de O. Gomes et al. 2020). Although the need for more sustainable pest control measures is largely recognized and increasingly practiced, synthetic pesticides are still used to a large degree.

1.2. Insecticide resistance evolution

Insecticides are substances that are used to kill pest insects (Stephenson et al. 2006) and they are important for food security and for the control of dangerous disease vectors. But the excessive use of insecticides has led to severe environmental consequences (Le Goff & Giraudo 2019) and to the development of resistance mechanisms in many different insect species (Sparks & Nauen 2015). Resistance means that the lethal effect of the insecticide on the target species decreases as the selection pressure from insecticide application results in the selection of individuals that are genetically predisposed to survive insecticide exposure (Panneton et al. 2001; Heckel 2012). Although chemical insecticides are usually novel synthetic compounds, target insect species are often able to develop resistance just a few years after introduction of a new insecticide (Hawkins et al. 2018; Le Goff & Giraudo 2019). This rapid adaptation happens under the strong selective pressure from the insecticide and is thus the result of evolutionary processes (Hawkins et al. 2018). For over a century, development of insecticide resistance has led to an dynamic arms race between scientists and pest control on the one side and insect pests and evolutionary processes on the other side (Heckel 2012; Le Goff & Giraudo 2019; Blanton & Peterson 2020). Greater understanding of the process of insecticide resistance development can improve risk assessment and management strategies for insecticide resistance as well as enhance general understanding of adaptive evolutionary processes resulting from novel substances and changing environments (Hawkins et al. 2018).

The main adaptive mechanisms behind insecticide resistance are usually changes in the insect genome that reduce sensitivity to the compound by causing target-site modification, or metabolic resistance through up-regulation of degrading enzymes to enhance metabolic breakdown, or enhancement of drug excretion (efflux). (Després et al. 2007; Rivero et al. 2011; Hawkins et al. 2018; Le Goff & Giraudo 2019). Although these mechanisms behind insecticide resistance development might have been altered through selective pressure caused by insecticides, it is unlikely that these complex detoxification mechanisms of insects have developed *de novo* within the short time frame of human pesticide use (Hawkins et al. 2018). Many insecticides are analogous to phytotoxins and the mechanisms behind insecticide resistance development may have evolved in herbivorous insects as a reaction to plant defense compounds (Hardy et al. 2018; Hawkins et al. 2018). In this way, insects might be pre-adapted to handle insecticides due to the selection pressure they are facing from their host plant defense compounds or pathogen toxins (Gordon 1961; Hawkins et al. 2018). The speed of insecticide resistant development in a herbivorous

insect species furthermore seems to be mediated by factors such as insect diet range and the chemical similarity between insecticide and phytochemicals in the insect diet (Crossley et al. 2021). There seems to be a link between adaptation to host plant toxins and insecticide resistance evolution that needs to be addressed to understand insecticide resistance development. One suggestion to explain this link, may be the involvement of gut microbiota that could aid the insect both in pesticide resistance and development on different host plant species (A. Bras, in prep).

1.2.1. Insecticide resistance and the impact of gut microbiota

Except for the above-mentioned adaptations evolved by the insect body, there are ecological factors that can cause insecticide resistance development. One factor that has gained more attention in recent years is the symbiotic relationship between insects and their gut microbiota, the community of microorganisms present in the insect gut (Marchesi & Ravel 2015). The insect gut microbiota can include protists, fungi, archaea and bacteria, but most organisms in the guts of most insect species are bacterial species. Those bacterial communities vary largely in total size, composition, location and function within the gut (Engel & Moran 2013). The evolutionary success of insects partly seems to depend on their mutualistic relationship with beneficial gut microorganisms. The gut bacteria might assist their insect host with nutrient uptake, protection against predators, parasites and pathogens, increasing thermal tolerance as well as helping with intra- and interspecific communication (Russell & Moran 2006; Engel & Moran 2013; Douglas 2015; Bosch & Welte 2017). Gut microbiota can increase host resistance to parasite invasion through nutrient competition, niche occupation or immune priming (Engel & Moran 2013; Douglas 2015). Gut symbionts can have beneficial effects for its host helping it to overcome diverse stresses, or the effects can be neutral or even adverse for example increasing the effect of toxins (Skaljac et al. 2018; Liu & Guo 2019) or reducing development and fecundity (Thakur et al. 2015). In the insect gut microbiota many beneficial or pathogenic microorganisms with similar or conflicting needs, interact with each other competitively or synergistically (Hamdi et al. 2011). The insect gut has coevolved with symbiotic microorganisms (Engel & Moran 2013) and offers advantages for colonizing microorganisms such as access to nutrients and protection from environmental stressors outside the insect body (Douglas 2015).

Insect gut microbiota seems to harbor bacterial symbionts that can aid their host insects with insecticide detoxification (Blanton & Peterson 2020). This phenomenon was first shown 50 years ago (Boush & Matsumura 1967) and up until today, gut symbiont-mediated resistance has been found in

more systems. For example for *Anopheles arabiensis* (Barnard et al. 2019), *Bactrocera dorsalis* (Cheng et al. 2017), *Bombyx mori* (Chen et al. 2020) and *Spodoptera frugiperda* (Almeida et al. 2017). However, resident gut bacteria have also been shown to increase susceptibility to insecticides in some lepidopteran species (Broderick et al. 2009) and it seems as if the effect of the gut microbiota for insecticide resistance seems to vary depending on the insect species, bacterial strains and composition of the gut microbial community (Xia et al. 2018; Liu & Guo 2019). Gut microbiota can improve resistance in the insect host by detoxification or degradation of toxins, by mediating host fitness, or by changing gene expression of themselves or the host insect (Liu & Guo 2019). Insecticide resistance might even be acquired instantly by horizontal transfer through infection with an insecticide-degrading soil bacteria (Kikuchi et al. 2012).

In summary, there is a growing scientific interest to investigate gut symbiont-mediated insecticide resistance. And more knowledge in that field of interest might be useful for the development of new pest management strategies as well as bioremediation practices (Blanton & Peterson 2020). However, more research is needed to gain a better understanding of symbiont-mediated resistance processes. One area of interest is the gut microbiota of lepidopteran species, which is an important phytophagous insect group with many severe pest species. The knowledge about the lepidopteran gut microbiota is small in general and there are several knowledge gaps. The extent of dependency on gut microbiota and the strength of the host-microbiota association are controversial for lepidopteran species (Staudacher et al. 2016; Hammer et al. 2017; Paniagua Voirol et al. 2018). Some results seem to suggest that gut microbiota is mostly acquired through the diet and that the host-microbiota association is weak (Phalnikar et al. 2018) – in that case, the influence of gut microbiota might be a link to understand the development of cross resistance between host plant adaptation and insecticide resistance. In that context it is also interesting that there are hints that the gut microbiota can enhance or decrease the effect of plant defenses on the host insect (Mason 2020). There seems to be substantial variations in lepidopteran gut microbiota composition and diversity depending on species, life stage, habitat, diet and interindividual bacterial transfer (Chen et al. 2016; Staudacher et al. 2016; Hammer et al. 2017; Paniagua Voirol et al. 2018). Therefore, more research is needed for different lepidopteran species to gain a broader understanding of the lepidopteran gut microbiota and its potential mediating effect on insecticide resistance.

1.2.2. Potential fitness costs of insecticide resistance and the influence of gut microbiota

Resistance evolution usually is expected to come with a cost. Coping with insecticides requires energy, resource allocation and possibly reduces efficiency of biological functions and insecticide resistance can therefore be costly with respect to insect fitness (Després et al. 2007; Kliot & Ghanim 2012). Fitness costs of insecticide resistance development on life-history traits and fecundity are often attributed to resource-based trade-off effects where the mechanisms behind insecticide resistance, such as overproduction of detoxification enzymes, compete with other biological functions over important resources that are needed for other vital biological processes (Rivero et al. 2011; Liu et al. 2021). Fitness costs of resistance are common, and they are often even seen in the absence of insecticides. Thus, it is not only costly for the insect to detoxify insecticides when it is exposed to them, but resistant insects seem to have some adaptations that always take energy from other important biological traits. And fitness costs for resistant strains compared to susceptible strains in the absence of insecticides are well documented for different insecticides and different species (Carriere et al. 1994; Boivin et al. 2001; Gassmann et al. 2009; Shi et al. 2020; Ullah et al. 2020; Garlet et al. 2021), even for *Spodoptera* species (Abbas et al. 2012, 2014; Okuma et al. 2018; Garlet et al. 2021; Liu et al. 2021). The type of fitness cost that arises from resistance evolution seems to depend on the specific resistance mechanisms developed by the insect (Smith et al. 2021). The degree of fitness costs associated with insecticide resistance development is influenced by environmental and ecological factors such as host plant species and its phytotoxins, access to refuges, degree of insecticide exposition, entomopathogens and intraspecific competition (Gassmann et al. 2009; Raymond et al. 2011).

Fitness costs of insecticide resistance might even be mediated by gut microbiota. The disadvantages of insecticide resistance development might be enlarged or reduced through host symbionts (Pietri & Liang 2018). One example for increase in fitness costs of resistance through gut bacteria was seen for the mosquito *Culex pipiens*. Infection with *Wolbachia* induced additional costs of resistance on some life-history traits for a resistant *C. pipiens* strain (Duron et al. 2006). A mediating effect on fitness costs for insecticide resistant strains was also suggested for the diamondback moth, *Plutella xylostella* (Li et al. 2019). That a gut symbiont might increase insecticide resistance, but also reduce insect fitness in resistant strains can be seen as an example for trade-off effects of symbiotic relationships. In mutualistic relationships, there is often a trade-off between gaining an advantage and the need to invest resources. Mutualistic relationships might

be beneficial in times of scarcity or crisis, but disadvantageous under perfect environmental conditions in the absence of danger (Frederickson et al. 2012). It has been shown that the advantage of bacterial symbionts on the fitness of aphids varied with temperature and bacterial strain (Russell & Moran 2006) Benefits for aphid fitness were enlarged if the aphids were exposed to heat shock, and some bacterial strain even reduced survival after heat shock compared with uninfected aphids (Russell & Moran 2006). Hence, although mutualistic relationships with microbial communities can have many advantages for insects, there might also be some costs associated with this mutualism. Gut microbiota can for example raise susceptibility to virus infection and mortality in a larval host compared to control group without gut microbiota (Jakubowska et al. 2013). It has also been found that higher symbiont density might cause higher costs for the host without increasing the benefits for the host (Parker et al. 2021). It has been shown that the cereal weevil *Sitophilus* reduces the amount of gut symbionts and recycles nutrients from them, when the benefit of symbiosis is no longer greater than the cost of maintaining the gut symbiont (Vigneron et al. 2014). However, it has also been found for *Speyeria mormonia* that the costs and benefits of different gut symbionts balance each other, creating no net cost for the insect (Ravenscraft et al. 2019).

That insecticide resistance often comes with fitness costs for the resistant strain compared to the susceptible strain might be a reason why insecticide resistance does not necessarily become completely established in field populations (Kliot & Ghanim 2012). Gut microbiota can stimulate immune response in the host, but even plant defenses and insecticides are capable of increasing insect immune response creating the potential for counteractive, additive or synergistic effects of microbiota, diet and insecticide exposure (Mason 2020). Benign resident bacteria can become opportunistic and damaging to its host due to disruptions and alterations in the gut microbial community (dysbiosis) caused by insecticide toxins (Broderick et al. 2009; Thakur et al. 2015; Pandey & Rajagopal 2017). This effect varies between Lepidopteran species depending on the composition of their gut microbial community (Broderick et al. 2009). Gathering knowledge on the fitness costs of developing resistance to insecticides is important for planning more appropriate integrated pest management strategies and for estimating the pace of insecticide resistance development (Kliot & Ghanim 2012).

1.3. Aims and research questions

More knowledge about the costs of insecticide resistance development and the potential role of the insect gut microbiota mediating resistance, costs and consequences of insecticide exposure is needed to gain more insight into the ecology behind resistance development and to refine pest management strategies. Moreover, knowledge about the effect of gut microbiota on insecticide resistance development is in general still lacking for lepidopteran species. Although many Lepidoptera species are economically important agricultural pests, the knowledge about the bacteria associated with lepidopteran species and their influence on their host insect is still insufficient (Paniagua Voirol et al. 2018).

The aim of this study was thus first to investigate (I) if the gut microbiota affects insecticide resistance to the synthetic pyrethroid Cypermethrin in lab strains of the polyphagous moth and crop pest *Spodoptera littoralis*. In a second step, it was studied (II) if exposure to Cypermethrin affected life history traits, development and fecundity of surviving *S. littoralis* larvae following exposition and if these potential consequences of insecticide exposition were mediated by the gut microbiota. And finally, it was studied (III) if there were any fitness costs of insecticide resistance development on life history traits, development and fecundity of *S. littoralis* and if these costs were mediated by the gut microbiota. To address these research questions, insecticide-resistant and insecticide-susceptible strains of *S. littoralis* larvae with and without damaged gut microbiota were exposed to the insecticide Cypermethrin and their survival, development and fecundity following insecticide exposition were studied.

The African cotton leaf worm (Noctuidae, Lepidoptera), *Spodoptera littoralis* (Boisduval), was used as the model species. *S. littoralis* is a nocturnal polyphagous moth with a wide host plant range (Lopez-Vaamonde 2009). *S. littoralis* is also a severe agricultural pest (EPPO 2021) that has developed resistance against several insecticides (Lopez-Vaamonde 2009). For *S. littoralis*, some research has been done concerning detoxification through protein alteration and enzymes (Hilliou et al. 2021). However, to my knowledge, no research has focused on symbiont-related detoxification processes in *S. littoralis* and the influence of its gut microbiota on insecticide resistance development.

2. Material and Method

The experiments were performed with an insecticide resistant and an insecticide susceptible laboratory strain of *Spodoptera littoralis* at the Swedish University of Agricultural Sciences (SLU) in Alnarp. The laboratory strain originates from Egypt and has been bred at the SLU laboratory for several years. To select for resistance, the resistant strain had been exposed during the 3rd larval instar to Cypermethrin over 6 generations. The selection pressure applied was 30% of survival. The susceptible strain had been exposed during the 3rd larval instar to a control solution over 6 generations (Bras, A. pers. commun.).

2.1. Study species *Spodoptera littoralis*

The African cotton leaf worm, *Spodoptera littoralis* (Boisduval), (Noctuidae, Lepidoptera) is a polyphagous moth with a very wide range of host plants, varying from grasses and legumes to fruit trees (Lopez-Vaamonde 2009). *S. littoralis* is native to semi-arid and subtropical habitats in pre-Saharan Africa and is one of the most severe agricultural lepidopteran pests within its range, attacking many economically important crops all year round (Lopez-Vaamonde 2009; EPPO 2021). Adult *S. littoralis* are nocturnal and despite the rather short flight range of 1.5 km, the species can be spread widely in international trade chains as eggs and larvae on plant material (Lopez-Vaamonde 2009). *S. littoralis* has extended its range around the Mediterranean Sea and is seen as a potential pest for greenhouses in Northern Europe (Lopez-Vaamonde 2009). Insecticide resistance of *S. littoralis* against several classes of insecticides has been known for decades and different management strategies have been tested (EPPO 2021). The *S. littoralis* gut microbiota is dominated by bacteria and composition and density of gut microbiota seems to vary during the insect's lifetime (Chen et al. 2016).

2.2. *S. littoralis* rearing

Eggs and larvae were kept in a climate chamber with 25°C, 60% relative humidity and a light/dark period of 12:12 hours. Adults were kept in another climate chamber with 25°C, 50% relative humidity and a light/dark period of 16:8 hours. Larvae were fed *ad libitum* with an artificial potato-based diet (recipe in Appendix 1). Adults were also fed *ad libitum* and offered sugar (beet sugar) that was diluted with tap water on a cotton ball. Eggs were collected from mating boxes and disinfected with formaldehyde fumes for 30 minutes. Afterwards, egg batches were placed on a freshly harvested cotton leaf inside a plastic rearing box with a mesh covered air window in the lid (Figure 1A). Larvae hatched after 2-3 days (Figure 1B).

Figure 1. A) Preparing rearing boxes for S. littoralis larvae and cutting out egg batches from the paper from the mating box. B) Newly hatched S. littoralis larvae on cotton leaf and pieces of diet. (Pictures by Sarah Heithausen)

From the newly hatched larvae 100 individuals were chosen at random per treatment and placed into a new rearing box (Figure 2). Two boxes were made with larvae from the resistant strain and two boxes were made with larvae from the susceptible strain. In both strains, the larvae were either fed with the regular diet or with the regular diet including antibiotics to eradicate the gut microbiota (see 2.3). The food was cut into 1.5 g pieces and placed directly into the box (Figure 3A). New pieces of food (several 1.5 g pieces, depending on number of larvae in the box, ca 150 mg per larvae) were placed into the rearing boxes 2-3 times per week. As a replicate, three days after the first batch and in the same manner, a second batch of 400 newly hatched larvae were evenly sorted into four different treatments (susceptible strain/ antibiotic food, susceptible strain/ control food, resistant strain/

antibiotic food, resistant strain/ control food). Hence, two replicate boxes of larvae were reared for each treatment.

Figure 2. The different containers used for Spodoptera littoralis rearing and the different experiments in my study.

Larvae were reared in groups, except for during the exposition experiment (see 2.5). After the exposition experiment, the surviving larvae from each treatment were reared together again until pupation and fed with the same diet as before the exposition. The larvae from the antibiotic treatment continued to receive antibiotic food until pupation and the larvae from the control diet treatment continued to receive standard food until pupation.

Figure 3. A) S. littoralis larvae feeding on 1.5 g pieces of artificial diet. B) S. littoralis larvae in a rearing box before pupation. (Pictures by Sarah Heithausen)

Before pupation, paper was offered to the larvae as a place to pupate (Figure 3B). Pupae were sexed and around 20 female pupae from each treatment, if possible, were chosen at random for mating and fecundity experiments (2.7). Female pupae were kept individually in eclosion cups (Figure 2) and then paired with a male for the rest of their lives inside a mating box (Figure 2). The remaining adults from each treatment (Figure 4) were kept in groups for the rest of their lives in rearing boxes, sorted after sex and treatment.

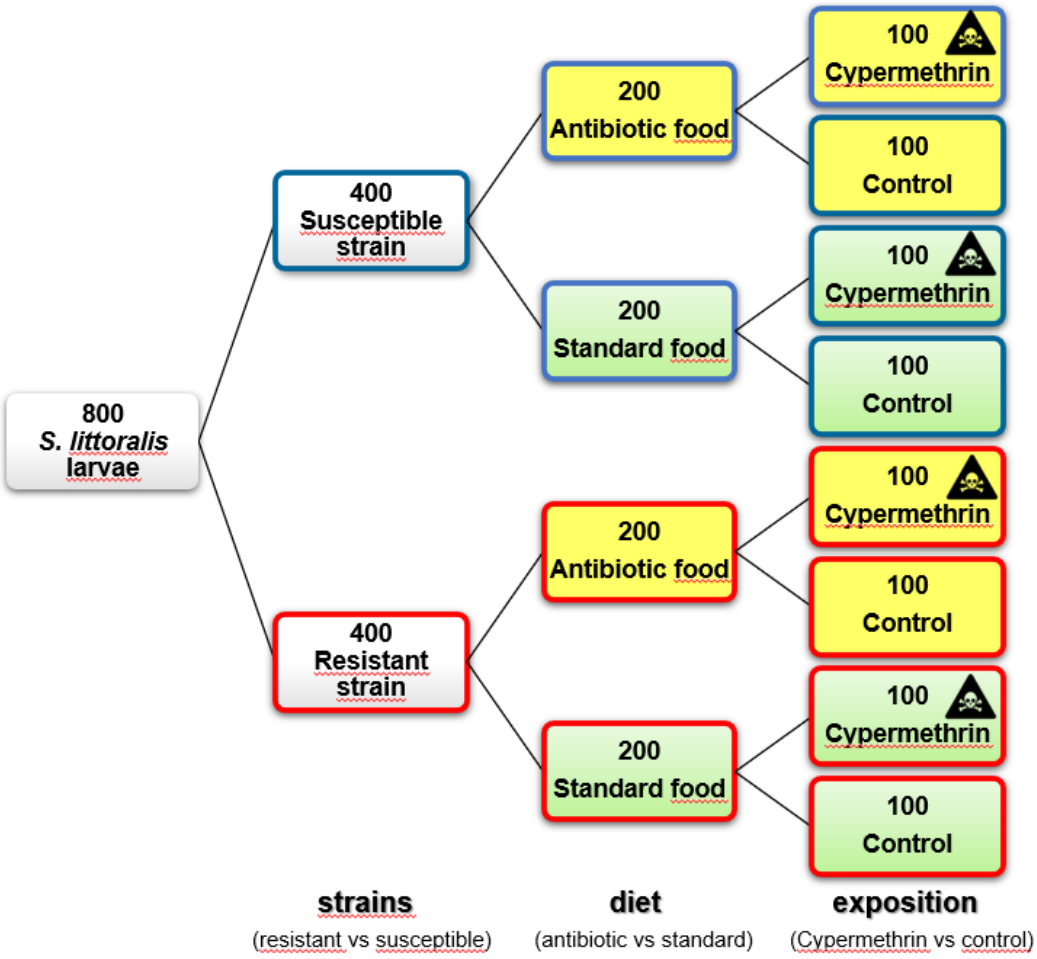

Figure 4. Experimental design and total number of S. littoralis larvae in the eight different treatments (2 replicates combined). The number in each tile represents the total number of S. littoralis larvae in that treatment. Of the 800 S. littoralis larvae in my study, 400 belonged to the susceptible strain (blue frame) and 400 belonged to the resistant strain (red frame). In each strain, 200 individuals received antibiotic diet (yellow background) and 200 individuals received standard food without antibiotics as control diet (green background). Finally, each of these four different treatments resulting from the different combinations of strain and diet was either exposed to the insecticide Cypermethrin (skull symbol) or to a control exposition (no skull).

2.3. Manipulation of *S. littoralis* gut microbiota through antibiotic diet

To assess the effect of *S. littoralis* gut microbiota on survival following insecticide exposition, development and fecundity, the gut microbiota was reduced in one treatment and left intact in a control treatment. A pilot study was conducted (Appendix 2) to develop a method for manipulation of the larval gut microbiota through addition of antibiotics to the *S. littoralis* diet. The aim of the pilot study was to test if adding antibiotic solution to larval food could affect larval gut microbiota or insect immediate survival and to find an effective combination of antibiotics to use in the subsequent study. Following the results of the pilot study (Appendix 2), an antibiotic cocktail including the two antibiotics Streptomycin sulfate (CAS-number 3810-74-0) and Ampicillin (CAS-number 69-52-3) was prepared. To do so, 200 mg Streptomycin were mixed with 50 ml MilliQ water and 200 mg Ampicillin were mixed with 50 ml MilliQ water. 35 ml from each of these two solutions was added directly to 1 liter of cooled down, liquid standard *S. littoralis* diet (Appendix 1) before it solidified. This antibiotic food was given to all larvae belonging to the experimental treatments that required manipulation of the gut microbiota. The larvae in those treatments were fed with antibiotic food for each meal from hatching to pupation. The larvae in the control condition that required no experimental manipulation of the gut microbiota were fed with standard *S. littoralis* diet without antibiotic cocktail for each meal from hatching to pupation. Adults from all treatments were fed with sugar solution without any antibiotics.

2.4. Larval dissection for manipulation control

To evaluate if the manipulation of *S. littoralis* larval gut microbiota through antibiotic diet was successful, 20 randomly chosen larvae from each treatment were dissected (2 x 4 larvae from each treatment from Batch A and 3 x 4 larvae from each treatment from Batch B as replicates). The extracted larval guts were diluted in autoclaved MilliQ water and plated on a culture medium to assess if the larvae that were fed with antibiotic food had less culturable bacteria in their guts than the larvae from the control condition.

Dissections were performed under a lamina hood in sterile conditions. Two hours before dissection, larvae were chosen randomly from each of the four treatments (resistant with standard diet, resistant with antibiotic diet, susceptible with standard diet, susceptible with antibiotic diet) and kept in small cups without food to be starved before dissection. Directly before

dissection the cups with the larvae were put on ice for a few minutes to stun the larvae. Afterwards, each larva was first dipped into a beaker with 95% ethanol and then into a beaker with autoclaved Milli-Q water. The larvae were dissected using two forceps that were sterilized with 95% ethanol between dissection of the different larvae. For each treatment and replicate, four larval guts were extracted and placed into a 1.5 ml Eppendorf tube filled with 1 ml of autoclaved Milli-Q water. The Eppendorf tube was vortexed for approximately 30 seconds and then 100 µl from the solution were transferred to another 1.5 ml Eppendorf tube filled with 900 µl autoclaved Milli-Q water. In this way a dilution series was performed, transferring 1/10th of the solution as many times as necessary to reduce the number of bacterial colonies eventually growing on the culture medium in order to make counting by eye feasible (Pepper & Gerba 2015). Results from the pilot study had shown that a thousand-fold dilution (10^{-3}) was necessary for larval guts from the control treatment, while no dilution series was necessary for the antibiotic treatment (Figure 5).

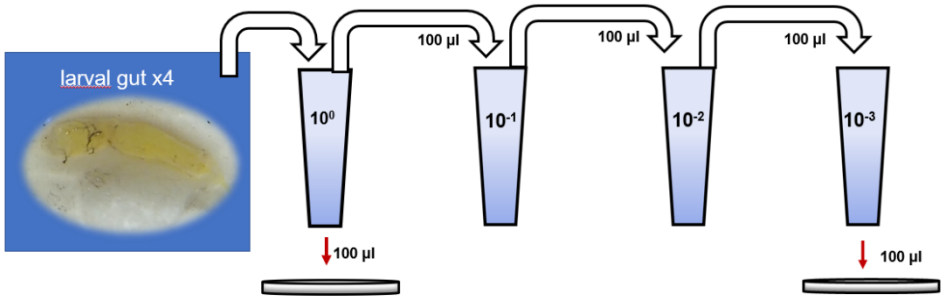

Figure 5. Dilution series of larval gut solution after dissection. 4 larval guts from the same treatment were put into 1 ml autoclaved MilliQ water and vortexed. For the antibiotic treatment 100 µl from that gut solution were plated onto a culture medium on a petri dish. However, further dilution was needed for the control treatment to make counting by eye of bacterial colonies later growing on that culture medium feasible. Larval gut solution from the control treatment was diluted 1000-fold before being plated onto the culture medium.

100 µl from each of the different dilutions were pipetted and spread on petri dishes with LB Agar (500 ml milliQ water + 20 g LB Agar) according to the spreading technique (Pepper & Gerba 2015). After the petri dishes had been inoculated with *S. littoralis* gut extract solution, they were incubated for 72 hours at 30°C in an incubator. After 72 hours the number of colonies on each plate was counted and the number of colony forming units (CFU) (Pepper & Gerba 2015) was calculated for the samples from the different treatments. CFU/ml was calculated with the formula:

$$number\ of\ colonies\ counted\ x\ 10^{d} / 0{,}1\ ml = \text{CFU/ml}$$

(The d in 10^{d} represents the number of dilution steps used in the dilution series.)

2.5. Insecticide exposition and assessment of survival

To assess the influence of *S. littoralis* gut microbiota on survival of insecticide exposition, 7-day old, 3rd instar larvae from the four treatment groups (susceptible strain with antibiotic diet, susceptible strain with control diet, resistant strain with antibiotic diet, resistant strain with control diet) were either exposed to the insecticide Cypermethrin or to autoclaved MilliQ water as control. Hence, the experiment finally consisted of eight different treatments (Figure 4).

During the exposition, small petri dishes (Figure 2) were equipped with a piece of 0.15 g standard *S. littoralis* food (Appendix 1). In the insecticide treatment, 50 µl of a solution of Cypermethrin + acetone + water (concentration 260 ng/ µl) was pipetted onto the food and in the control condition, 50 µl of a solution of water + acetone was pipetted onto the food. Acetone was added to the insecticide treatment to dissolve the Cypermethrin and thus also added the control treatment to control for any effects the acetone itself may have on larval survival. The applications were done under a fume hood and the pieces of food were left to dry there for approximately one hour. After this time, one *S. littoralis* larva was placed in each petri dish next to the piece of food and the petri dishes were closed with a lid. For each treatment, 50 small petri dishes were placed into one plastic box (Figure 6) and left in a climate chamber (25°C, 60% RH and 12:12 hours light/dark period) for 72 hours. Afterwards, larval survival was documented. The surviving larvae from each treatment were reared together receiving the same diet as before the insecticide exposition (either with or without antibiotics). Three days later the insecticide exposition was replicated with 50 more larvae from each treatment in the same manner.

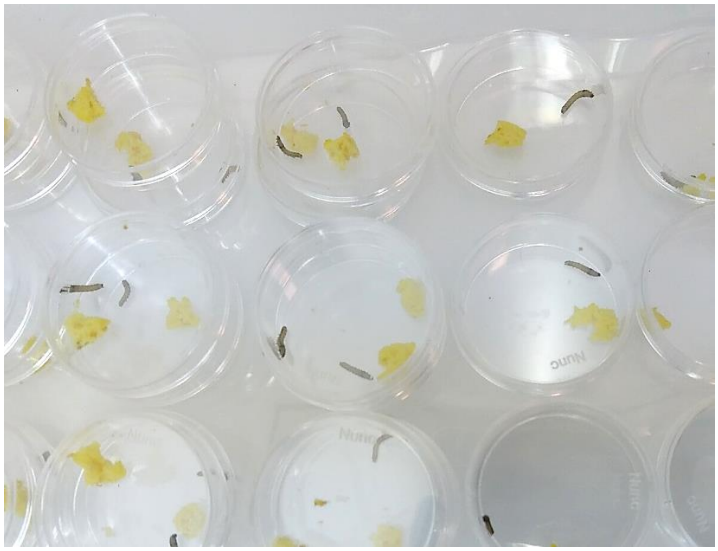

Figure 6. S. littoralis larvae during exposition experiment. (Picture by Sarah Heithausen)

2.6. Costs of insecticide resistance and effects of insecticide exposure on *S. littoralis* development

In order to understand if evolution of pesticide resistance comes with a cost and if gut microbiota can mediate these costs, as well as if resistance level and gut microbiota can mediate the effects of insecticide exposure, insects' performance throughout their life was studied. Larval development was followed until adulthood for the surviving larvae after the insecticide exposition, and several life-history traits were measured and compared between the eight different treatment groups (Figure 4). In addition, a mating and fecundity experiment was performed with a subset of the individuals (see 2.7 for details). Due to an uneven distribution of mortality following insecticide exposition, the number of individuals in the different treatments was unbalanced in the measurement of life-history traits (Table 1).

Table 1. Total number of individuals in the eight different treatments for the different measurements following the exposition experiment (both replicates combined).

Treatments			Number of individuals for measurements			
Strain	Diet	Exposition	Pupation rate of larvae	Larval development time, larval growth rate, sex ratio of pupae, pupal weight, and eclosion rate		Female pupal development time, female adult life span and fecundity
				♀	♂	
susceptible	standard	insecticide	24	10	10	9
susceptible	standard	control	100	45	41	18
susceptible	antibiotic	insecticide	36	14	16	14
susceptible	antibiotic	control	100	41	46	19
resistant	standard	insecticide	55	23	24	18
resistant	standard	control	100	36	27	18
resistant	antibiotic	insecticide	88	37	43	19
resistant	antibiotic	control	100	25	24	20

As the larvae were reared in groups, no individual hatching dates could be recorded. However, the date of pupation was documented for each individual and important life-history traits such as first larvae that hatched, first larvae that pupated, first adult that eclosed were recorded for each rearing box, indicating the fastest performance for each treatment.

Rearing boxes with larvae close to pupation were checked every day for new pupae that were taken out and weighed. **Pupal weight** was recorded as well as **larval development time** (number of days from hatching to pupation). The hatching date for each box was observed and considered sufficient as estimate for development time. The collected data was even

used to calculate the **larval growth rate** (pupal weight in mg/larval development time in days) for each individual pupa. To measure **pupation rate** (survival from hatching until pupation) it was documented how many of the larvae that survived the exposition experiment pupated from each treatment. Newly pupated *S. littoralis* were sexed with the help of a microscope (see Appendix 3) to calculate **sex ratio** between male and female pupae for each treatment.

Eclosion rate (survival during pupation) was recorded by counting how many adults eclosed from the pupae in each treatment. Even **survival until adulthood following insecticide exposition** was documented, by observing how many of the larvae that survived the exposition experiment eclosed as adults. **Female adult life span** was assessed for the females that were used in the mating and fecundity experiment by documenting eclosion date as well as death date of each female. Adult life span was only recorded for these randomly selected individuals as it was impossible to document individual hatching and death dates for the female adults living in groups.

2.7. Costs of insecticide resistance and effects of insecticide exposure on *S. littoralis* fecundity

To investigate if evolution of pesticide resistance affects fecundity, and if gut microbiota could mediate potential effects on fecundity following insecticide exposure, a subset of individuals was chosen randomly from each treatment for a mating and fecundity experiment. To assess fecundity, a female and male from the same treatment were paired together in a cone-formed mating box (Figure 2) as soon as the female had eclosed. The walls and lid of each mating box were lined with paper (greaseproof paper of brand “ICA smörgåspapper”, made in Denmark) offering the females an oviposition site. On the bottom of each mating box a small plastic cup was placed containing a cotton ball saturated with sugar solution as *ad libitum* food for the adults. The mating box was placed in a climate chamber (25°C, 50% RH and 16:8 hours of light/dark period) until the natural death of the female. Every third day, each mating box was checked for eggs (Figure 7). If eggs were found, the number of egg batches was counted. Then the eggs were removed with a paper edge and weighed.

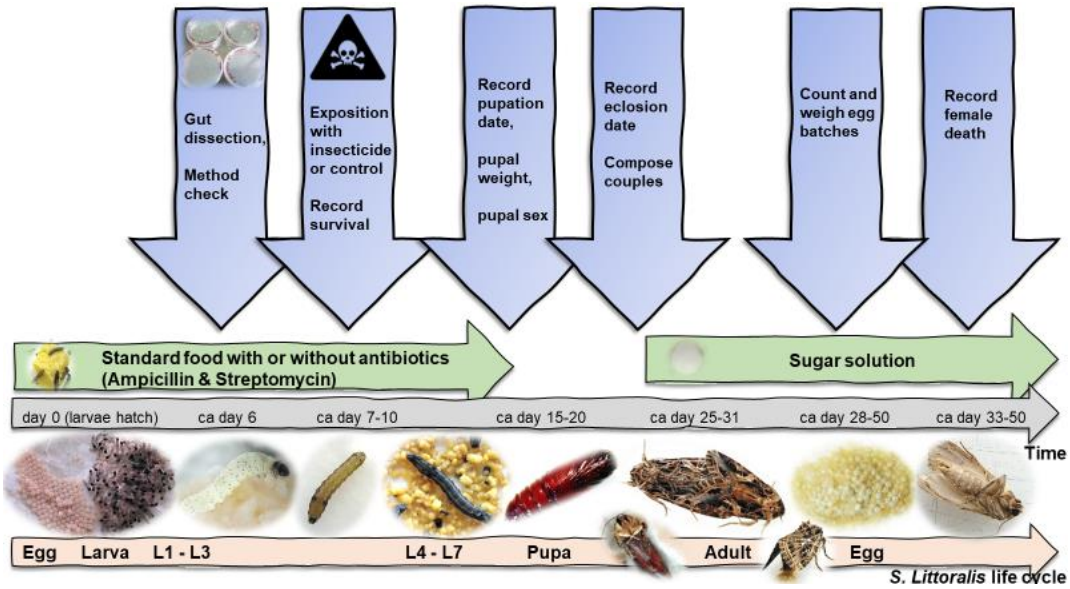

Figure 7. Overview of the different treatments and measurements implemented throughout the life cycle of the S. littoralis in my study. The vertical blue arrows show the measurements taken and the horizontal pink arrow shows the life stage of the insects during the measurements, while the horizontal grey arrow gives an estimate of the days passed since hatching. The horizontal green arrows show which food the insects were reared on during their life cycle.

2.8. Statistical analyses

Data was collected in Excel (Version 2018) and the statistical analyses were calculated with R 4.1.0 in R Studio (R Core Team 2021). Differences between the treatments in survival of insecticide exposition, pupation rate, eclosion rate and sex ratio were analyzed with generalized linear model, binomial logistic regression. This model was used since the data was on the nominal scale level. Dependent variables that were integrated as main factors in the model were strain, diet and exposition. Batch was included as an extra factor to correct for any differences between batches. Differences in larval growth rate, pupal weight, egg weight and number of egg batches were analyzed with ANOVAs. This analysis could be used since the data was ratio scaled and the residuals were normally distributed making the use of a parametric test possible. Dependent variables that were integrated as main factors in the ANOVA were strain, diet and exposition. As above, batch was included as an extra factor. A post hoc Tukey test was used to analyse differences between the treatments more closely. Differences in larval development time, female pupal development time and female adult life span were calculated with a non-parametric Kruskal Wallis test, since the residues were not normally distributed, prohibiting the use of ANOVA. Larval development time, larval growth rate, pupal weight and eclosion rate were analyzed separately for females and males.

3. Results

Dissection and cultivation of gut solution showed that antibiotic-treated larvae contained on average 123 times less bacterial colonies in the susceptible strain and 33333 times less bacterial colonies in the resistant strain compared to larvae from the same strain that had not received antibiotic treatment (Table 2).

Table 2. Results from gut dissection and cultivation of gut solution from randomly selected larvae from the four treatments. Each replicate included the guts of 4 randomly chosen larvae from each treatment. Gut solution from antibiotic-treated larvae included 100 – 493000 times less culturable colony forming units (CFU) per ml gut solution than the gut solution of larvae from the same strain that had not been treated with antibiotics. This result could be seen for both strains. In some cases, the gut solution had not been diluted enough before plating it and the colonies on the petri dish were too numerous to be counted (TNTC).

Treatment		CFU/ml							
Strain	Diet	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	mean	sd	se
susceptible	control	100	880	TNTC	TNTC	TNTC	490	551.54	390
susceptible	antibiotic	0	0	0	10	10	4	5.48	2.45
resistant	control	770000	810000	4930000	1410000	3080000	2200000	1790838	800887
resistant	antibiotic	0	30	10	0	290	66	125.82	56.27

Thus, it could be assumed as a premise for the subsequent experiments that the gut microbiota had been manipulated by the antibiotic diet.

3.1. Survival following insecticide exposition

The pattern of survival following exposition with either Cypermethrin or control compared over the eight different treatments was alike in both replicates (Appendix 4, table 18). In both replicates all larvae survived the control exposition (Figure 8).

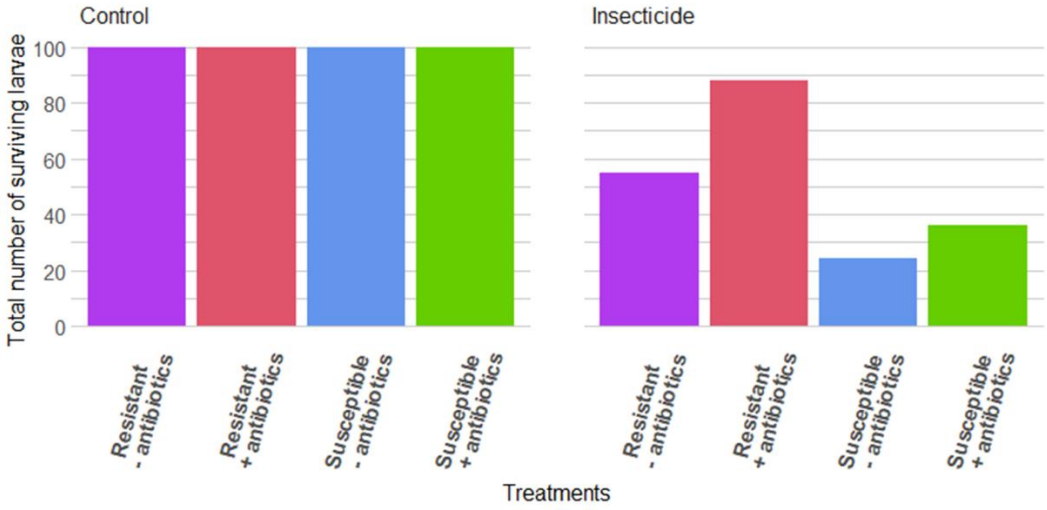

Figure 8. Total number of larvae that survived exposition experiments in each of the eight treatments. The number of surviving larvae from the 2 replicates were accumulated. Larvae were either exposed to the insecticide Cypermethrin or to a control. Larvae were either from the resistant or susceptible strain. Prior to the exposition experiments, larvae had either been treated with antibiotics (Streptomycin + Ampicillin) or not.

Logistic regression with generalized linear model including all larvae (n=800), the three main factors exposition, diet and strain and their interactions (Table 3) showed a highly significant batch factor (z= -5.85, p= 5.2×10^{-9}***). Larvae from batch A survived 1.21 times more often than larvae from batch B. Only the main factor exposition (insecticide *vs* control) had a statistically significant influence on larval survival following exposition (z= -5.24, p= 1.6×10^{-7}***). Larvae exposed to Cypermethrin died 1.97 times more often than larvae exposed to the control. The other two main factors strain (resistant *vs* susceptible) and diet (antibiotic *vs* standard) had no significant influence on larval survival following exposition. Neither had any possible interaction between the different main factors.

Table 3. Results for larval survival following exposition with either insecticide or control. The logistic regression with generalized linear model included all larvae from all treatments (n=800) and the main factors strain, exposition and diet as well as their interactions (represented with an x).

Factor	Estimate	Std. Error	z value	p value
Strain	-5.6×10^{-14}	1.01	0.00	1.00
Exposition	-3.92	0.75	-5.24	**1.6×10^{-7} *** **
Diet	-9.5×10^{-15}	1.01	0.00	1.00
Batch	-1.36	0.23	-5.84	**5.2×10^{-9} *** **
Strain x exposition	-1.43	1.06	-1.35	0.18
Strain x diet	2.3×10^{-14}	1.43	0.00	1.00
Exposition x diet	1.81	1.08	1.68	0.09
Strain x exposition x diet	-1.21	1.51	-0.80	0.42

Since all larvae survived the control exposition and the effect of exposition on following larval survival was highly significant, the larvae from the control exposition were excluded from the model and the effect of strain and diet on larval survival following exposition was calculated exclusively for larvae that had been exposed to the insecticide Cypermethrin (n=400). Logistic regression with generalized linear model including the two main factors strain and diet (Table 4) showed that both main factors as well as their interaction had significant influence on differences in larval survival (Figure 9).

Table 4. Results for larval survival following insecticide exposition. The logistic regression with generalized linear model included only insecticide-exposed larvae (n=400) and the main factors strain and diet as well as their interaction.

Factor	Estimate	Std. Error	z value	p value
Strain	-1.46	0.32	-4.53	**5.9×10^{-6} *****
Diet	1.84	0.37	5.02	**5.3×10^{-7} *****
Batch	-1.50	0.25	-6.09	**1.1×10^{-9} *****
Strain x diet	-1.23	0.49	-2.53	**0.0114 ***

Larvae from the susceptible strain were 2.38 times less likely to survive insecticide exposition than larvae from the resistant strain (z= -4.53, p= 5.9×10^{-6}***). Larvae that had been fed with antibiotic diet prior to insecticide exposition had a 1.57 times higher chance for survival than larvae that had been fed with control diet (z= 5.02, p= 5.3×10^{-7}***). There was also a significant strain x diet interaction (Figure 9). The survival-enhancing effect of antibiotic treatment prior to insecticide exposition was larger for larvae from the resistant strain compared to larvae from the susceptible strain (z= -2.53, p=0.0114*).

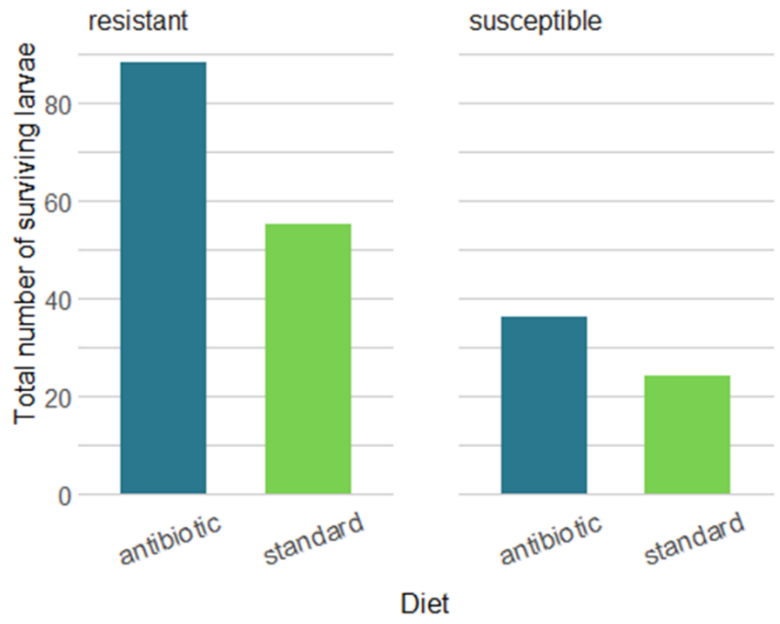

*Figure 9. Effect of strain and diet on larval survival following insecticide exposition (n=400). Larvae from the susceptible strain were less likely to survive than larvae from the resistant strain (z= -4.53, p= 5.9x10^{-6}***). Larvae that had been fed with antibiotic diet prior to insecticide exposition had a higher chance of survival than larvae that had been fed with standard diet (z= 5.02, p= 5.3x10^{-7}***). A significant interaction between the two main factors was found: The survival-enhancing effect of antibiotic treatment prior to insecticide exposition was larger for larvae from the resistant strain compared to larvae from the susceptible strain (z= -2.53, p=0.0114*).*

3.2. Development

3.2.1. Larval development

Female larval development time (number of days from hatching to pupation) differed significantly between the two batches (x^2 = 21.18, df = 1, p= 4.2x10^{-6}***) (Table 5). and between the different treatments (x^2 = 88.52, df = 7, p= 2.5x10^{-16}***) (Figure 10). Female larval development time differed significantly between the two strains (x^2= 36.31, df = 1, p= 1.7x10^{-9}***) and was on average 1.38 days shorter for the resistant strain. Female larval development time differed even significantly between the different expositions (x^2= 18.21, df = 1, p= 2.0x10^{-5}***) and was on average 1.13 days longer after insecticide exposition. Female larval development time was shorter for the resistant strain. Antibiotic treatment had no significant effect on female larval development (x^2= 2.58, df = 1, p= 0.108).

Table 5. Analyses of larval development showing the influence of the main factors strain (resistant vs susceptible), exposition (insecticide vs control) and diet (antibiotic vs standard) as well as their interactions on larval development time and larval growth rate. Larval development time was analyzed with a Kruskal Wallis test and larval growth rate was analyzed with an ANOVA.

Factor	Female larval development time	Male larval development time	Female larval growth rate	Male larval growth rate
Strain	x^2=36.31; df=1 **p= 1.7 x 10^{-9}***** resistant (n=121) $\bar{x}$=18.48 ± 1.7 (sd) susceptible (n=110) $\bar{x}$=19.86 ± 2.1 (sd)	x^2= 9.70; df=1 **p= 0.0018**** resistant (n=118) $\bar{x}$=18.64 ± 1.8 (sd) susceptible (n=113) $\bar{x}$=19.46 ± 2.1 (sd)	$F_{1,222}$= 28.24 **p= 2.6 x 10^{-7}***** resistant (n=121) $\bar{x}$=16.07 ± 3.9 (sd) susceptible (n=110) $\bar{x}$=17.89 ± 4.3 (sd)	$F_{1,222}$= 9.14 **p= 0.0028**** resistant (n=118) $\bar{x}$=15.75 ± 3.1 (sd) susceptible (n=113) $\bar{x}$=16.36 ± 3.0 (sd)
Exposition	x^2=18.21; df=1 **p= 2.0 x 10^{-5}***** insecticide (n=84) $\bar{x}$=19.86 ± 2.2 (sd) control (n=110) $\bar{x}$=18.73 ± 1.8 (sd)	x^2= 38.67; df=1 **p= 5.0 x 10^{-10}***** insecticide (n=94) $\bar{x}$=19.85 ± 1.7 (sd) control (n=137) $\bar{x}$=18.49 ± 1.9 (sd)	$F_{1,222}$= 16.41 **p= 7.1 x 10^{-5}***** insecticide (n=84) $\bar{x}$=18.18 ± 4.6 (sd) control (n=147) $\bar{x}$=16.22 ± 3.8 (sd)	$F_{1,222}$= 11.11 **p= 0.001**** insecticide (n=94) $\bar{x}$=16.75 ± 3.1 (sd) control (n=137) $\bar{x}$=15.57 ± 2.9 (sd)
Diet	x^2=2.58; df=1 p= 0.11 antibiotic (n=117) $\bar{x}$=18.84 ± 1.4 (sd) standard (n=114) $\bar{x}$=19.45 ± 2.5 (sd)	x^2= 2.19; df=1 p= 0.14 antibiotic (n=129) $\bar{x}$=18.78 ± 1.4 (sd) standard (n=102) $\bar{x}$=19.37 ± 2.5 (sd)	$F_{1,222}$= 3.47 p= 0.064 antibiotic (n=117) $\bar{x}$=16.69 ± 4.3 (sd) standard (n=114) $\bar{x}$=17.18 ± 4.1 (sd)	$F_{1,222}$= 2.27 p= 0.133 antibiotic (n=129) $\bar{x}$=15.90 ± 3.1 (sd) standard (n=102) $\bar{x}$=16.24 ± 2.9 (sd)
Batch			$F_{1,222}$= 23.47 **p= 2.4 x 10^{-6}*****	$F_{1,222}$= 24.77 **p= 1.3 x 10^{-6}*****
Exposition x strain			$F_{1,222}$= 18.21 **p= 2.9 x 10^{-5}*****	$F_{1,222}$= 22.40 **p= 4.0 x 10^{-6}*****
Exposition x diet			$F_{1,222}$= 2.72 p= 0.101	$F_{1,222}$= 3.09 p= 0.080
Strain x diet			$F_{1,222}$= 11.50 **p= 0.0008****	$F_{1,222}$= 4.65 **p= 0.032***
Exposition x strain x diet			$F_{1,222}$= 0.00 p= 0.997	$F_{1,222}$= 0.49 p= 0.487

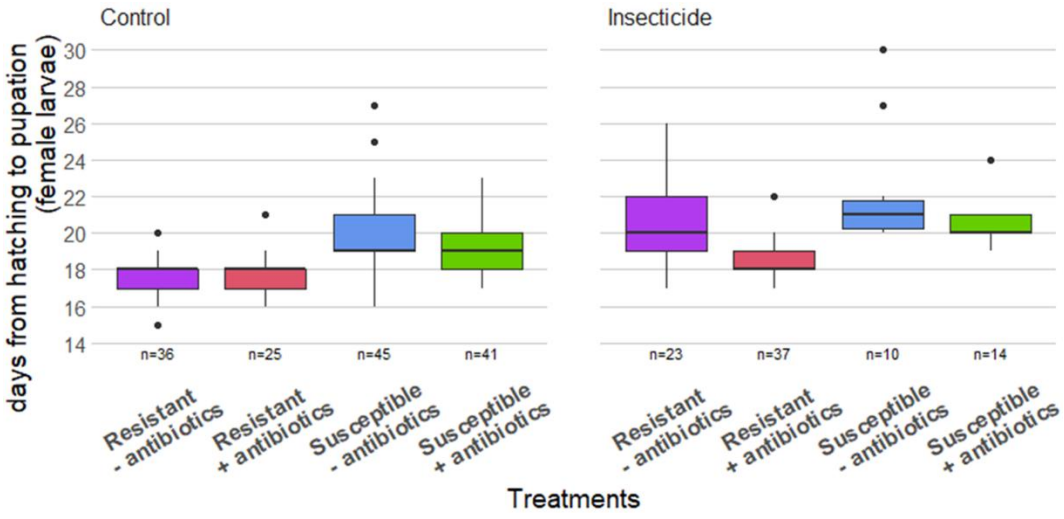

Figure 10. Female larval development time (number of days from hatching to pupation) for the different treatments. The larvae had either been exposed to the insecticide Cypermethrin or to a control. Larvae were either from a resistant or a susceptible strain and were fed with or without antibiotics added to their diet.

Male larval development time differed significantly between the two batches (x^2= 25.15, df = 1, p= 5.3x10^{-7}***) (Table 5) and between the different treatments (x^2= 83.40, df = 7, p= 2.8x10^{-15}***) (Figure 11). Male larval development time differed significantly between the two strains (x^2= 9.70, df = 1, p= 0.0018**) and was on average 0.82 days shorter for the resistant strain. Male larval development time differed even significantly between the different expositions (x^2= 38.67, df = 1, p= 5.0x10^{-10}***) and was on average 1.36 days longer after insecticide exposition. Antibiotic treatment did not affect male larval development time (x^2= 2.19, df = 1, p= 0.139).

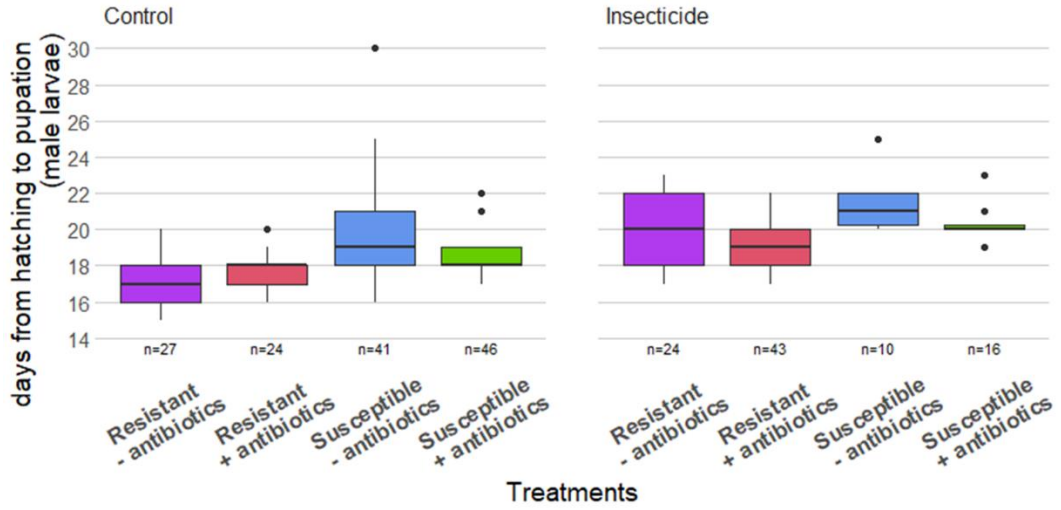

Figure 11. Male larval development time (number of days from hatching to pupation) for the different treatments. The larvae had either been exposed to the insecticide Cypermethrin or to a control. Larvae were either from a resistant or a susceptible strain and were fed with or without antibiotics added to their diet.

Female larval growth rate (pupa weight in mg/larval development time in days) differed significantly between the two batches ($F_{1,222}$= 23.47, p= 2.4x10^{-6}***) as well as between the different treatments ($F_{7,223}$= 9.40, p= 3.3x10^{-10}***) (Figure 12). Female larval growth rate was increased after insecticide exposition ($F_{1,222}$= 16.41, p= 7.1x10^{-5}***) (Table 5). Female larvae that had been exposed to Cypermethrin gained on average 1.96 mg more weight per day than control-exposed larvae. Female larval growth rate differed significantly between the two strains as well ($F_{1,222}$= 28.24, p= 2.6x10^{-7}***). Female larvae from the susceptible strain gained on average 1.82 mg more weight per day than resistant larvae. Antibiotic treatment tended to reduce female larval growth rate ($F_{1,222}$= 3.47, p= 0.064). Moreover, antibiotic treatment mediated the effect of strain on female larval growth rate as a highly significant interaction between strain and diet was

found ($F_{1,222}$= 11.50, p= 0.0008***). The effect of antibiotic treatment on female larval growth rate varied between the two strains (Figure 12).

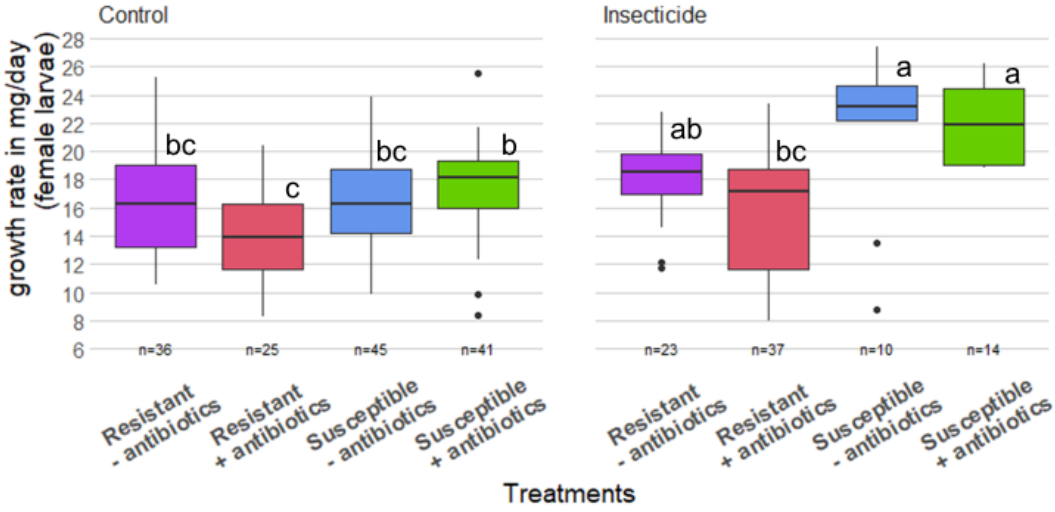

Figure 12. Differences in female larval growth rate (pupa weight in mg/larval development time in days) for the different treatments (n=231). Larvae had either been exposed to an insecticide (Cypermethrin) or to a control. Larvae were either from a resistant or susceptible strain and were either treated with antibiotics or not. The letters from a-c indicate significantly different groups in post hoc Tukey test (with 95% confidence level).

Male larval growth rate differed significantly between the two batches ($F_{1,222}$= 24.77, p= 1.3x10^{-6}***) as well as between the different treatments ($F_{7,223}$= 5.15, p= 1.9x10^{-5}***) (Figure 13). Male larval growth rate differed significantly between the two strains ($F_{1,222}$= 9.14, p= 0.003**) (Table 5). Male larvae from the susceptible strain gained on average 0.61 mg more weight per day than resistant male larvae. Male larval growth rate differed also significantly between expositions ($F_{1,222}$= 11.11, p= 0.001**). Insecticide-exposed male larvae gained on average 1.18 mg more weight per day compared with control-exposed male larvae. Antibiotic treatment had no significant influence on male larval growth rate ($F_{1,222}$= 2.27, p= 0.133). However, antibiotic treatment mediated the effect of strain on male larval growth rate. There was a significant interaction between strain and diet ($F_{1,222}$= 4.65, p= 0.032*) as the effect of antibiotic treatment on male larval growth rate varied between the two strains (Figure 13).

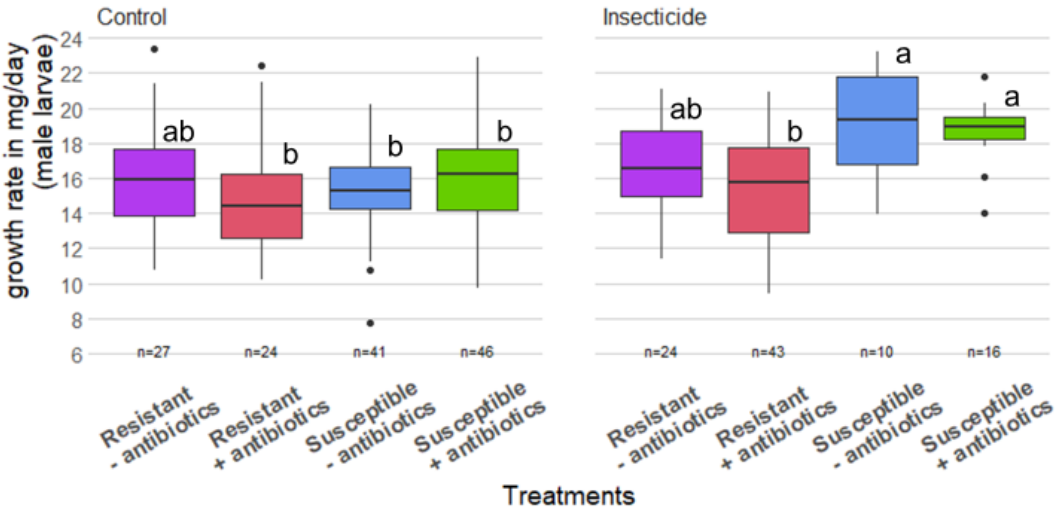

Figure 13. Differences in male larval growth rate (pupa weight in mg/larval development time in days) for the different treatments (n=231). Larvae had either been exposed to an insecticide (Cypermethrin) or to a control. Larvae were either from a resistant or susceptible strain and were either treated with antibiotics or not. The letters from a-b indicate significantly different groups in post hoc Tukey test (with 95% confidence level).

3.2.2. Pupation

Pupation rate was calculated for larvae that had previously survived the exposition experiment (n=603). Larvae from the resistant strain that had been treated with antibiotics had the highest pupation rate of all treatments after insecticide exposition, but the lowest pupation rate after control exposition (Figure 14, Table 1).

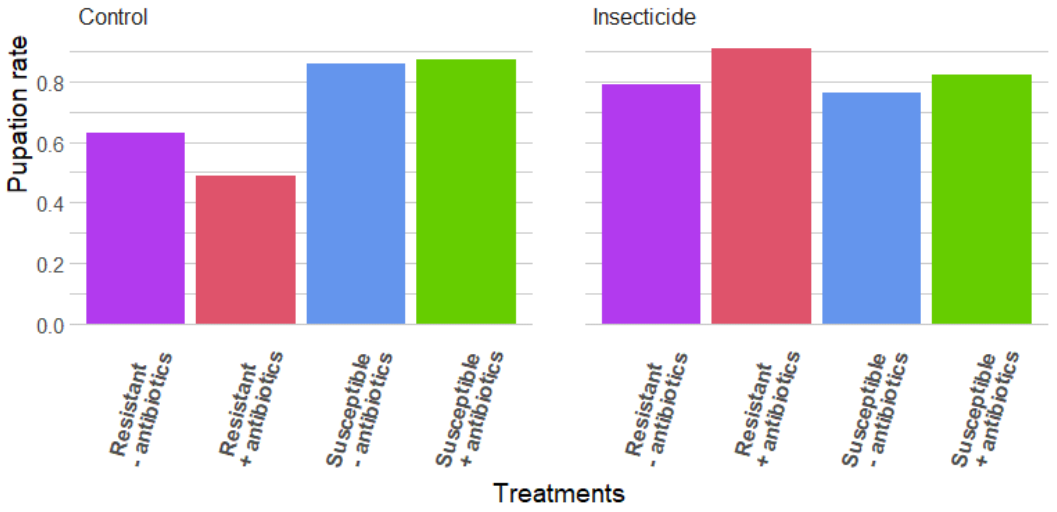

Figure 14. Pupation rate of larvae (n=603) that had previously survived exposition (either with the insecticide Cypermethrin or a control). Larvae were either from a susceptible or a resistant strain and had either been treated with antibiotics or not. The scale for pupation rate ranged from 0 (none of the larvae pupated) to 1 (all larvae pupated).

Pupation rate differed significantly between the two batches (z= -9.00, p < $2x10^{-16}$***) (Table 6). Larvae from the susceptible strain were 7% less likely to pupate than larvae from the resistant strain (z= 3.99, p=$6.6x10^{-5}$***). Pupation rate was 38% reduced after insecticide exposition compared to the control exposition (z= 2.37, p= 0.018*). Larvae that had been fed with antibiotic food were 12% more likely to pupate than larvae that had been fed with standard food (z= -2.32, p= 0.021*). Moreover, antibiotic treatment mediated the effect of exposition on pupation rate as a significant interaction was found between diet and exposition (z= 2.41, p= 0.016*). Antibiotic treatment increased pupation rate with 39% after insecticide exposition, but reduced pupation rate with 9% after control exposition.

Table 6. Pupation rate following exposition with either insecticide or control. The logistic regression with generalized linear model included all larvae that had previously survived exposition with the insecticide Cypermethrin or a control (n=603). The influence of the main factors strain, exposition and diet as well as their interactions on pupation rate was analyzed.

Factor	Estimate	Std. Error	z value	p value
Strain	1.56	0.39	3.99	**6.6×10^{-5} *** **
Exposition	1.14	0.48	2.37	**0.018***
Diet	-0.79	0.34	-2.32	**0.021***
Batch	-2.38	0.26	-9.00	**2×10^{-16} *** **
Strain x exposition	-1.91	0.84	-2.29	**0.022***
Strain x diet	0.88	0.55	1.60	0.110
Exposition x diet	1.60	0.67	2.41	**0.016***
Strain x exposition x diet	-1.83	1.12	-1.64	0.102

Another aspect of pupation that was analyzed was **sex ratio** of the pupae. Logistic regression with generalized linear model showed no significant influence of the main factors strain, exposition and diet or any of their interactions on pupal sex ratio (Table 7).

Table 7. Differences in sex ratio between the different treatments. Logistic regression with generalized linear model showed no influence of the main factors strain, exposition and diet or their interactions on sex ratio of pupae or adults.

Factor	Sex ratio pupae	Sex ratio adults
Strain	z= -0.58, p=0.56	z= -0.19, p=0.85
Exposition	z= -0.85, p=0.39	z= -0.09, p=0.93
Diet	z= -0.65, p=0.52	z= -0.49, p=0.63
Batch	z= 0.01, p=0.99	z= 0.75, p=0.45
Exposition x strain	z= 0.38, p=0.71	z= -0.01, p=0.99
Exposition x diet	z= 0.26, p=0.80	z= 0.34, p=0.73
Strain x diet	z= 0.08, p=0.94	z= 0.08, p=0.94
Exposition x strain x diet	z= -0.08, p=0.94	z= -0.26, p=0.79

Pupal sex ratio did not differ statistically significant between the different treatments (Figure 15).

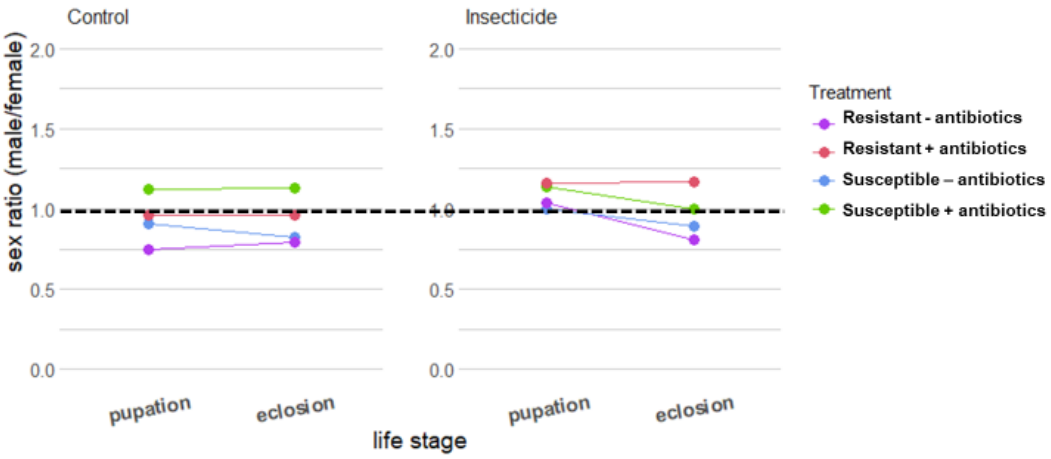

Figure 15. Sex ratio between male and female pupae and adults after pupation and eclosion for the different treatments. The insects had previously been either exposed to the insecticide Cypermethrin or to a control. The insects were either from a resistant or a susceptible strain and were fed with an artificial diet either with or without antibiotics (Streptomycin + Ampicillin). The black dashed line indicates the exact equilibrium between male and female insects. Data points above the dashed line show a surplus of male insects and data points below the dashed line show a surplus of female insects. Imbalance between the number of male and female insects increases in proportion to the distance to the dashed line.

Another aspect of pupation that was analyzed was **pupal weight** which was assessed separately with ANOVA for female (n=231) and male (n=231) pupae. **Female pupal weight** differed significantly between the two batches ($F_{1,222}$= 49.33, p= $2.6x10^{-11}$***) (Table 8) as well as between the different treatments ($F_{7,223}$= 17.20, p< $2x10^{-16}$***) (Figure 16). Female pupal weight was on average 16% higher after insecticide exposition compared to control exposition ($F_{1,222}$= 39.22, p= $1.9x10^{-9}$***). Female pupae from the susceptible strain were on average 16% heavier than female pupae from the resistant strain ($F_{1,222}$= 75.43, p= $8.3x10^{-16}$***). Antibiotic treatment reduced female pupal weight by 5% on average ($F_{1,222}$= 10.34, p= 0.002**). Moreover, antibiotic treatment mediated the effect of exposition and strain on female pupal weight as a significant interaction was found between the factors exposition and diet ($F_{1,222}$= 8.46, p= 0.004**) as well as between the factors strain and diet ($F_{1,222}$= 7.71, p= 0.006**). The effect of antibiotic diet on female pupal weight varied between the strains and depending on type of previous exposition.

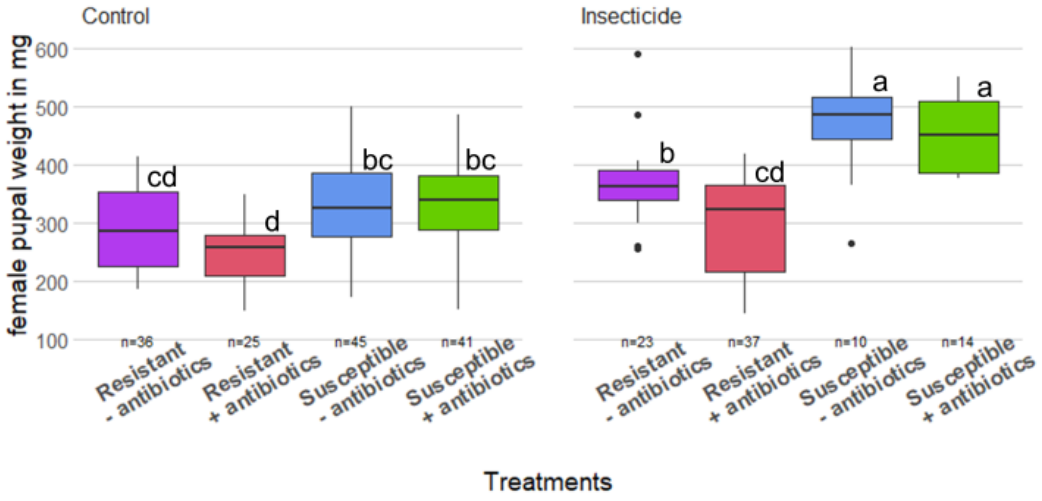

Figure 16. Mean female pupal weight for the different treatments (n=231). During the larval stage the pupae had either been exposed to the insecticide Cypermethrin or to a control. Pupae were either from a resistant or a susceptible strain and had during the larval stage either been fed with or without antibiotics (Ampicillin + Streptomycin) added to their diet. The letters a-d indicate classes of significant differences from post-hoc Tukey-test (with 95% confidence level).

Male pupal weight differed significantly between the two batches ($F_{1,222}$= 62.71, p= 1.1x10^{-13}***) (Table 8) as well as between the different treatments ($F_{7,223}$= 12.42, p= 2.1x10^{-13}***) (Figure 17).

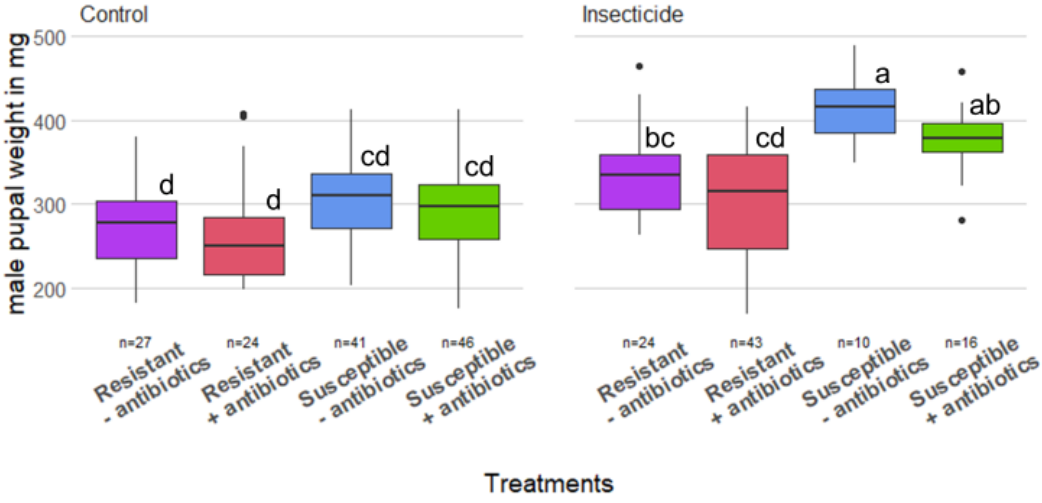

Figure 17. Mean male pupal weight for the different treatments (n=231). During the larval stage the pupae had either been exposed to an insecticide (Cypermethrin) or a control. Pupae were either from a resistant or a susceptible strain and had during the larval stage either been fed with or without antibiotics (Ampicillin + Streptomycin) added to their diet. The letters a-d indicate classes of significant differences from post-hoc Tukey-test (with 95% confidence level).

Male pupal weight was on average 14% higher after insecticide exposition compared to control exposition ($F_{1,222}$= 48.08, p= 4.4x10^{-11}***). Male pupae from the susceptible strain were on average 7% heavier than male pupae

from the resistant strain ($F_{1,222}$= 41.44, p= 7.4x10^{-10}***). Antibiotic treatment reduced male pupal weight by 4% on average ($F_{1,222}$= 9.92, p= 0.002**). Moreover, antibiotic treatment mediated the effect of strain on male pupal weight as a significant interaction was found between the factors exposition and diet ($F_{1,222}$= 8.02, p= 0.005**). The effect of antibiotic diet on male pupal weight varied depending on the type of previous exposition (insecticide *vs* control).

Table 8. Influences of the main factors strain, exposition and diet and their interactions on pupal weight and female pupal development time. Pupal weight was analyzed with an ANOVA and female pupal development time with a Kruskal Wallis test.

Factor	Female pupal weight	Male pupal weight	Female pupal development time
Strain	$F_{1,222}$=75.43 **p= 8.3 x 10^{-16}**** resistant (n=121) $\overline{x}$=298.0 ± 81 (sd) susceptible (n=110) $\overline{x}$=355.6 ± 95 (sd)	$F_{1,222}$= 41.44 **p= 7.4 x 10^{-10}***** resistant (n=118) $\overline{x}$=294.2 ± 66 (sd) susceptible (n=113) $\overline{x}$=318.1 ± 66 (sd)	x^2= 15.44, df=1 **p= 8.5 x 10^{-5}***** resistant (n=75) $\overline{x}$=4.09 ± 2.2 (sd) susceptible (n=60) $\overline{x}$=5.65 ± 2.0 (sd)
Exposition	$F_{1,222}$= 39.22 **p= 1.9 x 10^{-9}***** insecticide (n=84) $\overline{x}$=362.0 ± 102 (sd) control (n=147) $\overline{x}$=304.5 ± 80 (sd)	$F_{1,222}$= 48.08 **p= 4.4 x 10^{-11}***** insecticide (n=93) $\overline{x}$=333.5 ± 72 (sd) control (n=138) $\overline{x}$=287.4 ± 57 (sd)	x^2= 12.22, df=1 **p= 0.00047***** insecticide (n=60) $\overline{x}$=5.48 ± 2.1 (sd) control (n=75) $\overline{x}$=4.23 ± 2.2 (sd)
Diet	$F_{1,222}$= 10.34 **p= 0.00149**** antibiotic (n=117) $\overline{x}$=317.0 ± 93 (sd) standard (n=113) $\overline{x}$=334.0 ± 91 (sd)	$F_{1,222}$= 9.92 **p= 0.0019**** antibiotic (n=129) $\overline{x}$=299.9 ± 69 (sd) standard (n=102) $\overline{x}$=313.6 ± 64 (sd)	x^2= 3.04, df=1 p= 0.081 antibiotic (n=72) $\overline{x}$=4.40 ± 2.0 (sd) standard (n=63) $\overline{x}$=5.22 ± 2.3 (sd)
Batch	$F_{1,222}$= 49.33 **p= 2.61 x10^{-11}*****	$F_{1,222}$= 62.71 **p= 1.1 x 10^{-13}*****	x^2= 32.63, df=1 **p= 1.1 x 10^{-8}*****
Exposition x strain	$F_{1,222}$= 23.45 **p= 2.4 x 10^{-6}*****	$F_{1,222}$= 27.01 **p= 4.6 x 10^{-7}*****	
Exposition x diet	$F_{1,222}$= 8.46 **p= 0.004****	$F_{1,222}$= 8.02 **p= 0.005****	
Strain x diet	$F_{1,222}$= 7.71 **p= 0.006****	$F_{1,222}$= 0.49 p= 0.484	
Exposition x strain x diet	$F_{1,222}$= 0.60 p= 0.440	$F_{1,222}$= 0.00 p= 0.967	

Female pupal development time (number of days between pupation and eclosion), measured for females that had been randomly selected for fecundity trials (n=135), differed significantly between the two batches (x^2 = 32.63, df = 1, p= 1.1x10^{-8}***) (Table 8) as well as between the different treatments (x^2 = 42.86, df = 7, p= 3.6x10^{-7}***) (Figure 18). Resistant female pupae developed on average 1.4 days faster than susceptible pupae (x^2 =

15.44, df = 1, p= 8.5x10^{-5}***). Female pupal development time was on average 1.1 days longer after insecticide exposition compared to control exposition (x^2 = 12.22, df = 1, p-value = 0.00047***). Although female pupal development time did not differ significantly between pupae treated with or without antibiotics, antibiotic treatment tended to shorten female pupal development time by 0.6 days on average (x^2 = 3.04, df = 1, p= 0.081).

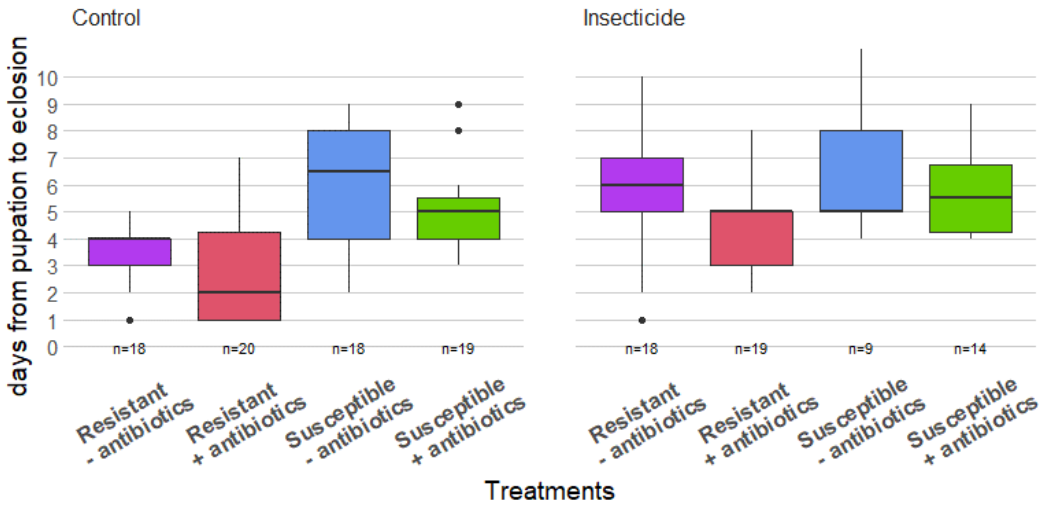

Figure 18. Mean female pupal development time (number of days between pupation and eclosion), measured for females that had been randomly selected for fecundity trials (n=135), is shown for the different treatments. During the larval stage the pupae had either been exposed to an insecticide (Cypermethrin) or a control. Pupae were either from a resistant or a susceptible strain and had during the larval stage either been fed with or without antibiotics (Ampicillin + Streptomycin) added to their diet.

3.2.3. Eclosion

Differences in **survival until adulthood** were analyzed for larvae that had survived exposition experiments (n=603) (Figure 19, Table 9). The rate of survival until adulthood was 13% lower for larvae from the resistant strain compared to larvae from the susceptible strain (z= 1.98, p= 0.048*). Whether the larvae had previously been exposed to an insecticide or a control did not significantly affect their survival until adulthood (z= 0.97, p= 0.335). Antibiotic treatment increased the rate of survival of the larvae until adulthood by 5% (z= -2.24, p= 0.025*). Moreover, antibiotic treatment mediated the effect of exposition and of strain on survival until adulthood as a significant interaction was found between diet and exposition (z= 3.37, p= 0.0007***) as well as between diet and strain (z= 3.09, p= 0.002**). After insecticide exposition, survival until adulthood was higher for larvae treated with antibiotics in both strains (Figure 19). But after control exposition

antibiotic treatment reduced survival rate for resistant larvae and increased survival rate for susceptible larvae.

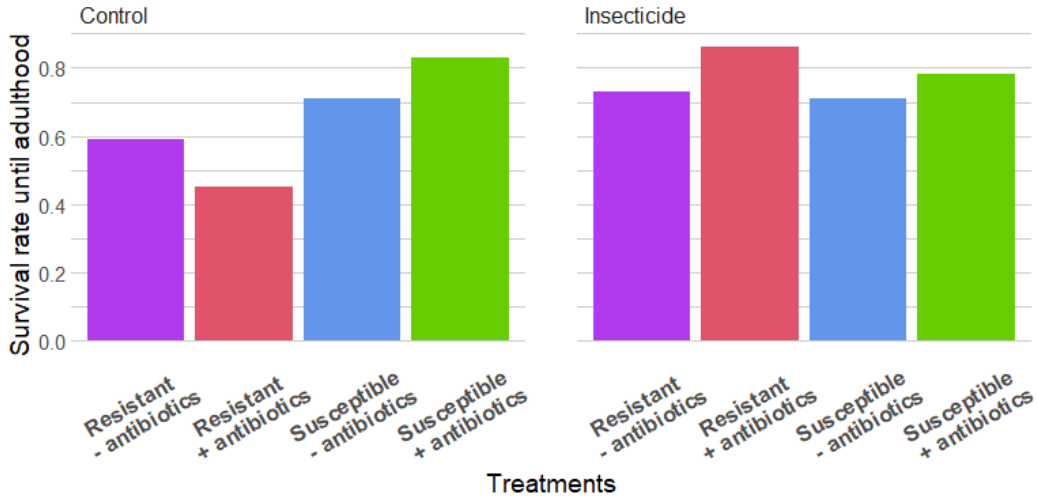

Figure 19. Eclosion rate of larvae that survived exposition experiment (n=603). Larvae had either been exposed to the insecticide Cypermethrin or to a control. Larvae were either from a resistant or a susceptible strain and had been fed with an artificial diet with or without antibiotics.

Eclosion rate of pupae (Appendix 4, Table 19) was analyzed separately for female (n=231) and male (n=231) pupae (Table 9). **Eclosion rate of female pupae** did not differ significantly between the different treatments (Figure 20, Table 9).

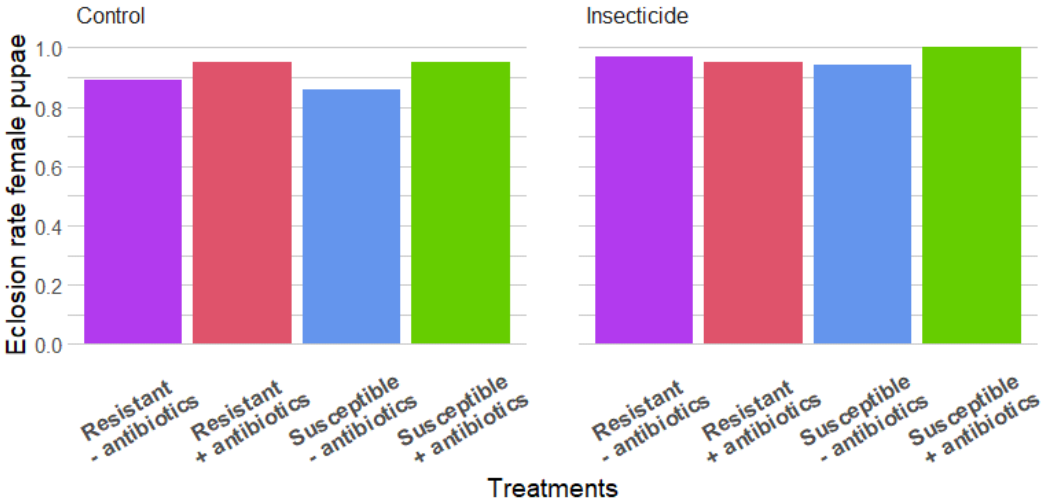

Figure 20. Eclosion rate of female pupae (n=231). During the larval stage, insects had either been exposed to the insecticide Cypermethrin or to a control. Pupae were either from a resistant or a susceptible strain and had previously been fed with an artificial diet with or without antibiotics (Streptomycin + Ampicillin). No statistically significant differences in eclosion of female pupae were found between the different treatments.

Results from analyses of survival until adulthood and eclosion rate of pupae are summarized in Table 9.

Table 9. Eclosion rates for larvae that had survived exposition and for female and male pupae. Eclosion rate was calculated for the larvae by dividing the total number of adults in a treatment with the total number of larvae in the same treatment. Eclosion rate for pupae was calculated by dividing the total number of adults in a treatment by the total number of pupae in the same treatment. Logistic regression with generalized linear model was used to analyze the influence of the main factors strain, exposition and diet and their interactions on eclosion rate.

Factor	Eclosion rate of surviving larvae (n=603)	Eclosion rate of female pupae (n=231)	Eclosion rate of male pupae (n=231)
Strain	z= 1.98 **p= 0.048*** resistant eclosion rate: 0.64 (220 adults / 343 larvae) susceptible eclosion rate: 0.77 (199 adults / 260 larvae)	z= -0.66 p= 0.51 resistant eclosion rate: 0.93 (113 adults / 121 pupae) susceptible eclosion rate: 0.92 (101 adults / 110 pupae)	z= -1.29 p= 0.20 resistant eclosion rate: 0.91 (107 adults / 118 pupae) susceptible eclosion rate: 0.87 (98 adults / 113 pupae)
Exposition	z= 0.97 p= 0.335 insecticide eclosion rate: 0.79 (161 adults / 203 larvae) control eclosion rate: 0.65 (258 adults / 400 larvae)	z= 0.56 p= 0.58 insecticide eclosion rate: 0.95 (80 adults / 84 pupae) control eclosion rate: 0.91 (134 adults / 147 pupae)	z= -1.97 **p= 0.048*** insecticide eclosion rate: 0.87 (81 adults / 93 pupae) control eclosion rate: 0.90 (124 adults / 138 pupae)
Diet	z= -2.24 **p= 0.025*** antibiotic eclosion rate: 0.72 (232 adults / 324 larvae) standard eclosion rate: 0.67 (187 adults/ 279 larvae)	z= 0.00 p= 1.00 antibiotic eclosion rate: 0.95 (111 adults / 117 pupae) standard eclosion rate: 0.90 (103 adults / 114 pupae)	z= -0.66 p= 0.51 antibiotic eclosion rate: 0.94 (121 adults / 129 pupae) standard eclosion rate: 0.82 (84 adults / 102 pupae)
Batch	z= -9.43 **p< $2x10^{-16}$***	z= -1.19 p=0.24	z= -3.46 **p=0.0005***
Exposition x strain	z= -1.33 p=0.184	z= -0.27 p=0.79	z= 1.18 p=0.24
Exposition x diet	z= 3.37 **p=0.00075***	z= -0.05 p=0.24	z= 2.05 **p=0.04***
Strain x diet	z= 3.09 **p=0.002****	z= 0.89 p=0.96	z= 1.75 p=0.08
Exposition x strain x diet	z= -2.52 **p=0.012***	z= 0.014 p=0.99	z= -2.04 **p=0.04***

Eclosion rate of male pupae differed significantly between the two batches (z= -3.46, p= 0.0005***) and was significantly reduced after insecticide exposition (z= -1.97, p= 0.048*) (Table 9, Figure 21). Antibiotic treatment mediated the effect of exposition and strain on eclosion rate of male pupae as a significant interaction was found between diet and exposition (z= 2.05, p= 0.041*) and even between diet, strain and exposition (z= -2.04, p= 0.04*). The effect of antibiotic treatment on eclosion rate of male pupae varied between strains and depending on the type of previous exposition (insecticide or control). Antibiotic treatment increased eclosion rate after insecticide exposition in both strains (Figure 21). After control exposition however, antibiotic treatment increased eclosion rate for the susceptible but not for the resistant strain.

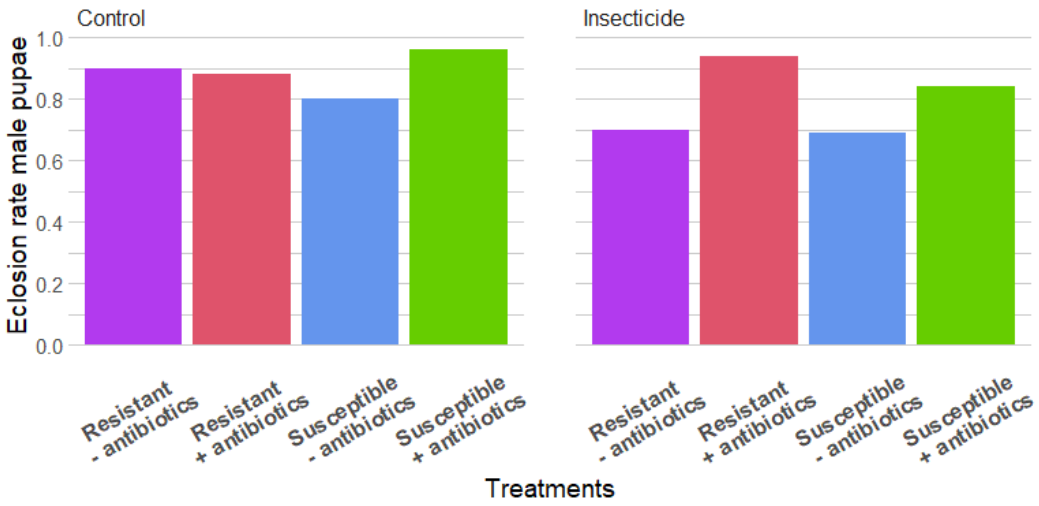

Figure 21. Eclosion rate of male pupae (n=231). During the larval stage, insects had either been exposed to an insecticide (Cypermethrin) or a control. Pupae were either from a resistant or a susceptible strain and had previously been fed with an artificial diet with or without antibiotics.

An overview of the total number of individuals from each treatment that survived exposition, pupated and eclosed as adults (Figure 22) shows that survival was influenced by previous exposition (insecticide *vs* control) and strain. After control exposition, the total number of individuals surviving until adulthood was higher for the susceptible strain. After insecticide exposition however, survival until adulthood was higher for the resistant strain. While antibiotic treatment increased the total number of susceptible adults both after control and insecticide exposition, the effect of antibiotic treatment on survival of resistant insects varied depending on exposition. After control exposition antibiotic treatment decreased the total number of resistant individuals surviving until adulthood, while the survival of antibiotic-treated individuals was increased after insecticide exposition.

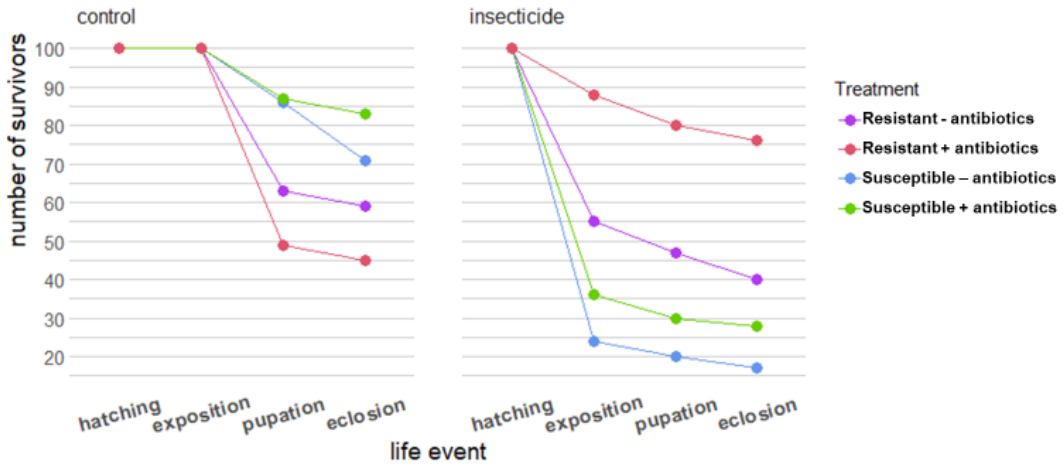

Figure 22. Timeline of survival of the original 100 larvae from each treatment following exposition, pupation and eclosion.

3.2.4. Female adult life span

Female adult life span did not differ significantly between the different treatments (x^2 = 12.30, df=7, p=0.912) (Figure 23) or between the two batches (x^2 =0.005, df=1, p=0.942). Female adult life span did not differ between the two strains (x^2= 3.81, df=1, p= 0.051) or between the two expositions (x^2 = 0.17, df=1, p=0.68). And antibiotic treatment had no effect on female adult life span either (x^2 = 1.07, df=1, p= 0.301).

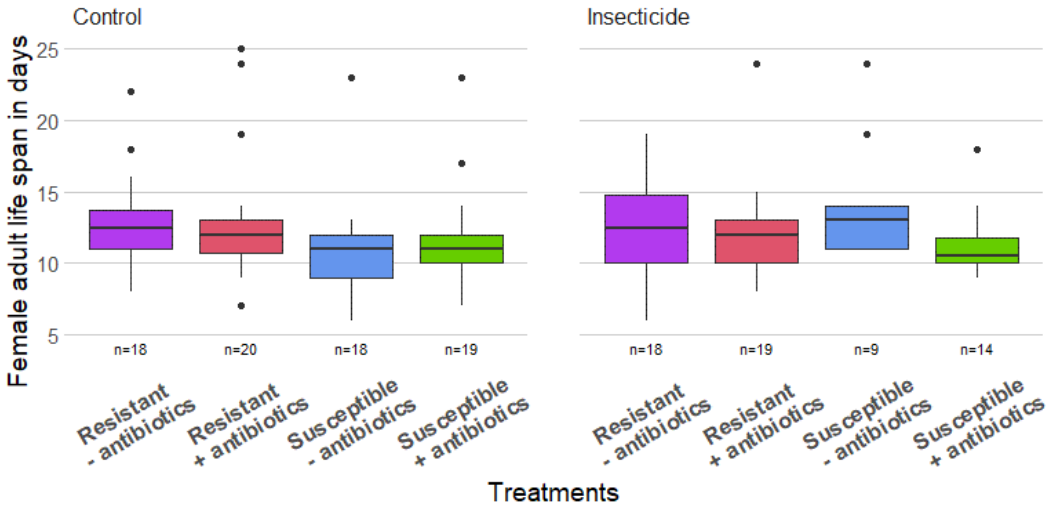

Figure 23. Female adult life span for the different treatments. During the larval stage, the insects had either been exposed to an insecticide (Cypermethrin) or a control. Insects were either from a resistant or a susceptible strain and as larvae had been fed with an artificial diet with or without antibiotics (Streptomycin + Ampicillin).

3.3. Fecundity

Female fecundity (total number and weight of egg batches laid per individual female) differed significantly depending on strain and exposition (Table 10). **Total egg weight** was significantly increased after insecticide exposition ($F_{1,125}$= 15.02, p= 0.0002***) and was on average 21% higher than after control exposition (Figure 24). Total egg weight differed also significantly between the two strains ($F_{1,125}$= 30.65, p= $1.7x10^{-7}$***) and was on average 26% higher for the susceptible strain. Antibiotic treatment had a slight tendency to reduce total egg weight ($F_{1,125}$= 3.14, p= 0.079) but did not mediate the effect of exposition or strain on total egg weight. Finally, female pupal weight had a highly significant influence on total egg weight ($F_{1,125}$= 35.30, p= $2.6x10^{-8}$***).

Table 10. Influence of the main factors strain, exposition and diet and their interactions on female fecundity (total weight and number of egg batches collected from each female) was analyzed with ANOVA for a subset of females from each treatment (n=135).

Factor	Egg weight per female in mg	Egg batches per female (total number)
Strain	$F_{1,125}$= 30.65 **p= 1.7 x 10^{-7}****	$F_{1,125}$= 15.51 **p= 0.00014*****
	resistant (n=75) $\bar{x}$=100.4 ± 51.0 (sd)	resistant (n=75) $\bar{x}$=9.2 ± 4.1 (sd)
	susceptible (n=60) $\bar{x}$=136.8 ± 47.2 (sd)	susceptible (n=60) $\bar{x}$=11.8 ± 4.5 (sd)
Exposition	$F_{1,125}$= 15.02 **p= 0.00017*****	$F_{1,125}$= 4.67 **p= 0.033***
	insecticide (n=60) $\bar{x}$=132.0 ± 47.0 (sd)	insecticide (n=60) $\bar{x}$= 11.2 ± 3.8 (sd)
	control (n=75) $\bar{x}$=104.2 ± 53.6 (sd)	control (n=75) $\bar{x}$=9.6 ± 4.8 (sd)
Diet	$F_{1,125}$= 3.14 p= 0.079	$F_{1,125}$= 1.49 p= 0.225
	antibiotic (n=72) $\bar{x}$=111.7 ± 58.6 (sd)	antibiotic (n=72) $\bar{x}$=10.0 ± 4.5 (sd)
	standard (n=63) $\bar{x}$=122.2 ± 44.2 (sd)	standard (n=63) $\bar{x}$=10.7 ± 4.4 (sd)
Batch	$F_{1,125}$= 2.60 p= 0.109	$F_{1,125}$= 0.01 p= 0.910
Female pupal weight	$F_{1,125}$= 35.30 **p= 2.6 x 10^{-8}*****	$F_{1,125}$= 6.25 **p= 0.014***
Exposition x strain	$F_{1,125}$= 0.83 p= 0.364	$F_{1,125}$= 1.89 p= 0.171
Exposition x diet	$F_{1,125}$= 2.08 p= 0.151	$F_{1,125}$= 0.06 p= 0.813
Strain x diet	$F_{1,125}$= 0.11 p= 0.745	$F_{1,125}$= 0.22 p= 0.637
Exposition x strain x diet	$F_{1,125}$= 0.001 p= 0.977	$F_{1,125}$= 1.42 p= 0.236

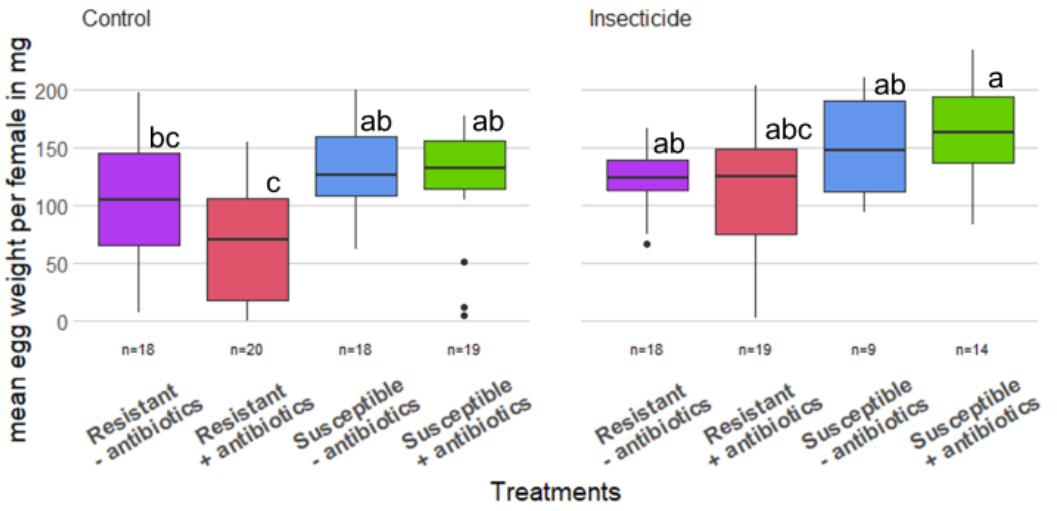

Figure 24. Differences in the average total egg weight per individual female for the different treatments. During the larval stage, the females had either been exposed to an insecticide (Cypermethrin) or a control. Females were either from a resistant or a susceptible strain and had during their larval stage been fed with artificial diet with or without antibiotics (Streptomycin + Ampicillin). The letters a - c indicate classes of significant differences from post-hoc Tukey-test (with 95% confidence level).

Total number of egg batches laid by each individual female was significantly increased after insecticide exposition ($F_{1,125}$= 4.67, p= 0.033*). Females oviposited on average 1.6 egg batches more after insecticide exposition than after control exposition (Table 10, Figure 25). Total number of egg batches differed also significantly between the two batches ($F_{1,125}$= 15.51, p= 0.0001***). Susceptible females oviposited on average 2.6 egg batches more than resistant females. Antibiotic treatment did neither affect the number of total egg batches nor mediated the effect of strain or exposition on the amount of total egg batches. However, female pupal weight had a significant influence on the total number of egg batches laid per female ($F_{1,125}$= 6.25, p= 0.014*).

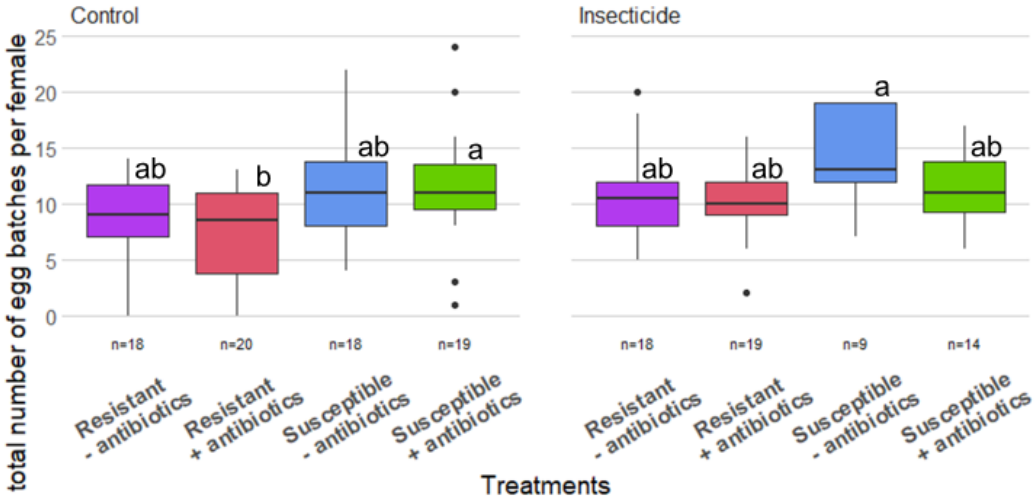

Figure 25. Differences in the total number of egg batches oviposited by each female. During the larval stage, the females had either been exposed to the insecticide Cypermethrin or to a control. Females were either from a resistant or a susceptible strain and had during their larval stage been fed with artificial diet with or without antibiotics (Streptomycin + Ampicillin). The letters a - b indicate classes of significant differences from post-hoc Tukey-test (with 95% confidence level).

3.4. Summary of the results

Results from the different analyses of the data from the different experiments and observations are summarized in table 11.

Table 11. Overview of results obtained from the different experiments and measurements showing the influence of the three main factors: Strain (resistant vs susceptible), Diet (antibiotic vs standard) and Exposition (insecticide vs control) on the studied life-history traits of S. littoralis. "No difference" means that no statistically significant difference in survival or performance of S. littoralis was found between the different treatments of a factor. A cell that is fully colored represents a statistically significant result and a cell that is light in color represents a tendency. The emojis in front of a colored background indicate whether a significant (or almost significant) effect is positive or negative for S. littoralis. A green cell with a smiling emoji shows which treatments were advantageous for the S. littoralis (for example higher survival rate or faster development compared to the other treatments). A red cell with a frowning emoji shows disadvantageous treatments to the individual (for example lower survival rate or slower development compared to the other treatments).

Experiment/Observation		Strain		Diet		Exposition	
		resistant	susceptible	antibiotic	standard	insecticide	control
Survival of insecticide exposition		☺	☹	☺	☹	☹	☺
Larval development time	♀	☺	☹	No difference		☹	☺
	♂	☺	☹	No difference		☹	☺
Larval growth rate	♀	☹	☺	☹ p=0.064	☺	☺	☹
	♂	☹	☺	No difference		☺	☹
Pupation		☺	☹	☺	☹	☹	☺
Sex ratio male/female		No difference		No difference		No difference	
Pupal weight	♀	☹	☺	☹	☺	☺	☹
	♂	☹	☺	☹	☺	☺	☹
Female pupal development time		☺	☹	☺ p=0.081	☹	☹	☺
Eclosion (from pupae)	♀	No difference		No difference		No difference	
	♂	No difference		No difference		☹	☺
Survival until adulthood of larvae after exposition (n=603)		☹	☺	☺	☹	No difference	
Fecundity (egg weight)		☹	☺	☹ p=0.079	☺	☺	☹
Female adult life span		No difference		No difference		No difference	

4. Discussion

Insect gut microbiota has been shown to have important functions for its host such as nutrient uptake or defense against pathogens (Engel & Moran 2013; Douglas 2015). It has recently been indicated that the gut microbiota might also have an effect on insecticide resistance for some insect species (Broderick et al. 2009; Kikuchi et al. 2012; Hilbeck et al. 2018; Skaljac et al. 2018; Xia et al. 2018). However, to my knowledge, little is known in general about the influence of gut microbiota on insecticide resistance in insects. And there is a lack of knowledge about the effect of *S. littoralis* gut microbiota on insecticide resistance development and potential trade off effects on life history traits of *S. littoralis* resulting from resistance development. To address this knowledge gap, I exposed larvae from a resistant and a susceptible *S. littoralis* lab strain to an insecticide (Cypermethrin) or a control. Prior to exposition and after exposition, the larvae were either treated with antibiotics (Streptomycin and Ampicillin) or not. After exposition, individual development and performance was recorded until death. Results showed that being resistant or not, being exposed to the insecticide or not and being treated with antibiotics or not were all factors that had significant influence on survival and several life history traits of *S. littoralis*.

4.1. Survival of insecticide exposition and the influence of the gut microbiota

As expected, more *S. littoralis* larvae from the resistant strain than from the susceptible strain survived exposition to the insecticide Cypermethrin. This was hardly surprising, because larvae from the resistant strain had been selected over six generations for resistance to Cypermethrin (A. Bras, unpublished data). However, in both strains, larvae survived insecticide exposition better when they had been fed with antibiotic diet prior to the insecticide exposition. That larvae with damaged gut microbiota survived to a higher degree suggests that the gut microbiota may not be involved in detoxification of Cypermethrin and thus not aid the insects in pesticide

resistance. Instead, the results suggest that there may be other physiological mechanisms than symbiotic gut bacteria that are responsible for lower susceptibility to Cypermethrin. That prior treatment with antibiotics can reduce mortality after insecticide exposition has already been found for *S. littoralis* (Hilbeck et al. 2018), other lepidopteran species such as *Helicoverpa armigera* (Visweshwar et al. 2015), *Ostrinia nubilalis* (Hilbeck et al. 2018), *Vanessa cardui*, *Manduca sexta*, *Pieris rapae*, and *Heliothis virescens* (Broderick et al. 2009) and for other insects such as *Anopheles arabiensis* (Barnard et al. 2019).

The negative effect of gut microbiota on survival of *S. littoralis* larvae following insecticide exposition may be due to the presence of pathogenic bacteria in the gut of the insects. Even apparently healthy insects might be infected with multiple pathogens (Rolff & Siva-Jothy 2003) and it has been found previously for *Lymantria dispar* (Broderick et al. 2009) and *Acyrthosiphon pisum* (Skaljac et al. 2018) that bacterial symbionts can increase susceptibility to insecticides. These pathogenic bacterial symbionts might stress the organism in some way making it more vulnerable to the toxic stress from the insecticide exposition. Moreover, inflammatory processes in the insect gut following insecticide exposition might even facilitate pathogenicity of opportunistic resident gut symbionts, thereby enhancing insecticide toxicity (Hilbeck et al. 2018; Mason 2020). The presence of so called pathobionts has been suggested. Bacteria that exist as commensal bacteria in an organism with functional immune system and healthy microbiota, but can become harmful in case of a disturbance in quantity or composition of gut microbial community (Drew et al. 2021). In *Spodoptera litura*, which is a close relative to *S. littoralis*, commensal gut bacteria, that are typically benign in an optimal environment, can begin having a positive or negative influence on its host in the presence of a stimulating stress condition such as inflammation of gut tissue (Pandey & Rajagopal 2017). Insecticide exposition might be such a stimulating stress condition and antibiotic treatment prior to insecticide exposition might reduce pathogenic or opportunistic bacterial gut symbionts. This reduction of pathogenic gut bacteria might have limited their stressful impact on the host and liberated resources that could have been used to cope with the toxic insecticide, thereby increasing the odds of survival for the *S. littoralis* host.

Even more interesting than the effect of the different strains by itself, my results hint towards a mediating effect of the gut microbiota on the effect of the different strains on survival of insecticide exposition since a significant interaction between the factors strain and diet was found. The survival-enhancing effect of the antibiotic diet was significantly larger in the resistant

strain compared to the susceptible strain. The two variables strain and diet thus seem to have a synergistic effect on survival of insecticide exposition. One explanation might be that the resistant strain might have adapted physiologically to insecticide detoxification through the selection process - an adaptation that might be lacking in the susceptible strain. When antibiotics were added to the diet pathogenic gut bacteria might have been reduced and resources that otherwise would have been used to contain these pathogenic bacteria could instead be used to increase detoxification of the insecticide even further. This possible reallocation of resources might have released the full resistance potential of the resistant strain and made insecticide detoxification in the resistant strain more efficient after antibiotic treatment. My results thus indicate that there is indeed a potential for resistance and gut microbiota to interact but there does not seem to be any resistant gut microbiota that aids the *S. littoralis* host in resistance, rather the interaction is in the opposite direction.

4.2. Influence of insecticide exposition on life history traits and potentially mediating role of gut microbiota

Insecticide exposition had significant influence on several life history traits of *S. littoralis* in my study. The effects could be positive or negative depending on life stage. Insecticide exposition increased larval growth rate, pupal weight, and fecundity, but decreased larval and pupal development time, pupation rate and male eclosion rate.

A mediating role of gut microbiota on the effect of insecticide exposition on pupation rate and survival until adulthood was indicated by a significant interaction between the factors diet and exposition. While antibiotic diet increased pupation rate and survival until adulthood after insecticide exposition, antibiotic diet reduced pupation rate and survival until adulthood after control exposition. Thus, gut microbiota seemed to affect pupation rate and survival until adulthood negatively only in case of prior insecticide exposition and seemed to be beneficial under control conditions. One reason for this context-dependent influence of gut microbiota might be that there seems to be a complex balance between costs and benefits of gut microbiota. The costs and benefits of a symbiosis for the host and the symbiont can change depending on the ecological context and abiotic factors such as environmental toxicity or the composition of the surrounding biotic community (Gerardo & Hurst 2017; Drew et al. 2021). Gut microbiota usually facilitates nutrient uptake for its host and aids in defense against

pathogens, which even seem to be important beneficial functions of *S. littoralis* gut microbiota (Chen et al. 2016; Shao et al. 2017). In this way, gut microbiota might favor host development, and antibiotic treatment might cause changes in gut microbial composition that impair nutrient uptake and other important functions of beneficial gut bacteria which could reduce pupation rate and survival until adulthood. However, even if gut microbiota might be beneficial to its host after control exposition, the symbiosis might become costly for the insect after insecticide exposition when disturbances in the gut microbial community might enable opportunistic bacteria to become pathogenic and disturb or slow down development. Pathogenic gut symbionts are costly to their host (Rolff & Siva-Jothy 2003), which is why antibiotics are often used in insect rearing to increase weight gain (Hammer et al. 2017). Hence, after insecticide exposition, a gut microbiota that has been reduced of pathogenic bacteria through prior antibiotic treatment might entail less stress for its host compared to an intact gut microbiota containing pathogenic bacteria.

Another disadvantage of insecticide exposition was that female pupal development time was prolonged after insecticide exposition, thereby possibly delaying mating and oviposition. However, pupal weight was increased after insecticide exposition, and this can be seen as an evolutionary advantage since a positive linear correlation between pupal weight and fecundity was found for *S. littoralis* in my study, but even in previous research (Abdel-Fattah et al. 1977). Not all life history traits were, however, affected by insecticide exposition. Insecticide exposition had no effect on pupal or adult sex ratio, female adult life span or survival until adulthood following insecticide exposition.

Interestingly, there were some sexual differences in how insecticide exposition affected *S. littoralis* in my study. While insecticide-exposition had no influence on eclosion for female pupae, eclosion rate was reduced for male pupae after insecticide exposition. Furthermore, an interaction between exposition and diet on eclosion was found for male pupae: The eclosion-increasing effect of antibiotic diet was larger after insecticide exposition than after control exposition. Such sexual differences in performance after insecticide exposition might be due to different investment or different life-history traits involved in mating or reproduction.

Insecticides may have further effects on mating and reproduction as female fecundity was affected by insecticide exposition. Mean weight and number of oviposited egg batches were increased after insecticide exposition. This might be partly explained by the increase in pupal weight after insecticide exposition. It has long been established that there is a positive linear correlation between pupal weight and fecundity. This

correlation has previously been found for *Spodoptera exigua* (Tisdale & Sappington 2001) as well as for *S. littoralis* (Abdel-Fattah et al. 1977). Insecticide exposition usually impairs insect fitness. However, my results show that growth and fecundity actually can be increased after insecticide exposition. A possible explanation for these findings might be a potential overcompensation of homeostatic processes in the insect body as a reaction to growth-inhibiting effects of the insecticide toxins (Jager et al. 2013; Guedes & Cutler 2014). A phenomenon, termed hormesis, has been observed that describes a reversed effect of a toxin where sublethal doses can be stimulatory while high doses are inhibitory or destructive (Cohen 2006; Jager et al. 2013; Pandey & Rajagopal 2017; Margus et al. 2019). The negative effects of insecticide exposition such as larval and pupal development time and decreased pupation rate might thus either be explained by actual damage to the organism caused by the insecticide or as trade-off effects resulting from compensatory or adaptive mechanisms used of the organism to cope with the insecticide toxins. Available resources might be redistributed, increasing performance in one trait (e.g. pupal weight) at the cost of decreasing performance in another (e.g. pupation rate) (Jager et al. 2013). Extent and character of these trade-off effects might, however, vary depending on environment and developmental stage (Jager et al. 2013). Such trade-off effects might explain why insecticide-exposition seemed to stimulate development in some life history traits of *S. littoralis* in my study such as larval growth, pupal weight and fecundity, but seemed to prolong larval and pupal development time and decrease pupation rate as well as eclosion rate. Positive effects on life history traits after insecticide exposition have been found for other insects as well. Colorado potato beetles (*Leptinotarsa decemlineata*) for example had higher adult survival and greater adult body mass when they had been exposed to a sublethal dose of deltamethrin as larvae compared to unexposed individuals (Margus et al. 2019). Such potentially stimulating effects of insecticide exposition might explain some of the observed effects of insecticide exposition on life history traits of surviving individuals in my study. However, the advantageous effects of insecticide exposition on some life history traits observed in my study could also be due to selection processes rather than hormesis effects (Guedes & Cutler 2014). Thus, the higher larval growth rate and pupal weight of insecticide-exposed individuals found in this study might be just an effect of the fittest individuals surviving exposition.

My results are in line with another study on the consequences of insecticide exposure on *S. littoralis* which found that exposition to LC_{50} doses of chlorantraniliprole and indoxacarb significantly prolonged *S. littoralis* larval and pupal development time and decreased pupation rate but

increased pupal weight (Moustafa et al. 2021). Further resembling my results that study also showed that insecticide exposition had no significant effect on sex ratio or eclosion rate. However, contrary to my results, no significant differences in female fecundity were found between insecticide-exposed and control-exposed *S. littoralis* (Moustafa et al. 2021). And also contrary to my results, in another study, *S. littoralis* pupal weight and fecundity were reduced after exposition to sublethal doses of the insecticide emamectin benzoate compared to a control exposition (Mokbel & Huesien 2020). It therefore seems that the costs of insecticide exposition for *S. littoralis* might vary depending on the type of insecticide used and that the costs of Cypermethrin exposition on *S. littoralis* life-history traits found in my study might not necessarily be generalized for other insecticides. Furthermore, I only observed life history traits for the generation of the insecticide-exposed individuals. However, insecticide exposition can have trans-generational effects, increasing larval survival and adult body mass of the next generation (Margus et al. 2019). Therefore, future research could investigate transgenerational effects of insecticide exposition for *S. littoralis* which might offer interesting knowledge gain for pest management strategies.

4.3. Costs of insecticide resistance on life history traits and influence of gut microbiota

Insecticide resistance development seemed to create benefits for *S. littoralis* survival and costs for *S. littoralis* development and fecundity. While the resistant strain had shorter larval and pupal development time and higher pupation rate, the rate of survival until adulthood was reduced. The resistant strain had lower larval growth rate, probably due to lower pupal weight which might also explain lower fecundity compared to the susceptible strain. Some life-history traits, namely sex ratio of pupae, eclosion rate and female adult life span were unaffected by strain and antibiotic treatment. In line with the costs for the resistant *S. littoralis* strain observed in my study, costs of resistance have also been found for many other insects such as *Choristoneura rosaceana* (Carriere et al. 1994), *Cydia pomonella* (Boivin et al. 2001) and *Culex pipiens* (Rivero et al. 2011). Even other *Spodoptera* species were shown to have costs of insecticide resistance such as longer development times and decreases in larval growth rate, larval survival rate, eclosion of healthy adults and fecundity found for resistant *S. litura* (Abbas et al. 2012, 2014), reduced larval and pupal weight, survival to adulthood and fecundity for resistant *S. frugiperda*

(Okuma et al. 2018; Garlet et al. 2021) and reduced fecundity for resistant *S. exigua* (Liu et al. 2021). Such costs of insecticide resistance development on life history traits and fecundity might be attributed to resource depletion for resistance mechanisms, such as overproduction of detoxification enzymes, that compete with other vital biological functions of the insect organism over important resources (Rivero et al. 2011; Pietri & Liang 2018; Liu et al. 2021). Interestingly, overproduction of detoxification enzymes has been found to be one of the main mechanisms of insecticide resistance in *S. littoralis* (Hilliou et al. 2021). Hence, adaptive mechanisms that have evolved in the resistant *S. littoralis* strain in my study might have used resources that might have been relocated from other important biological functions such as growth or fecundity. In case of insecticide exposition, the adaptative mechanism is costly, but might save the insect's life. However, in the absence of insecticides, these expensive adaptative mechanisms might be a disadvantage for resistant insects compared to susceptible insects. Although the evolution of resistance has been shown to be costly, it seems as if the amount and type of resistance cost might vary depending on the type of adaption mechanism that has evolved in the insect organism (Smith et al. 2021). This was observed in *Drosophila melanogaster* where two different target site mutations both created insecticide resistance, but only one of the target site mutations impaired insect fitness compared to a control population (Homem et al. 2020). Costs of resistance mechanisms such as target site mutations seem to depend on which other biological functions the target site provides for the organism (Homem et al. 2020). Hence, a better understanding of the type of resistance mechanisms that might have evolved in the resistant *S. littoralis* lab strain used in my study could be interesting for future studies.

Gut microbiota seemed to have a mediating effect on the cost of resistance since several significant interactions were found between strain and diet affecting larval growth rate, female pupal weight, and survival until adulthood. Antibiotic diet decreased larval growth rate in the resistant strain but enhanced growth rate slightly in the susceptible strain. The difference in female pupal weight between resistant and susceptible strain was enlarged after antibiotic treatment. The resistant strain survived slightly better until adulthood without antibiotics and the susceptible strain survived better with antibiotics. These different effects of the antibiotic treatment on the two different strains could be a hint that there might be variations in composition or function of the gut microbiota between the two strains. It is impossible for me to analyze these differences more in detail since my study design did not include identification of the different taxa or assessment of the composition or density of the gut microbiota. Life history traits such as larval

growth rate might not necessarily be influenced by one specific bacterial taxa alone, but by the overall composition of the gut microbiota, as shown in *Melitaea cinxia* (Ruokolainen et al. 2016). That costs of resistance development might be mediated by gut microbiota has been discussed before and the mediating effect of gut microbiota might vary depending on many factors such as composition of gut microbiota, insect diet, insect life stage or insect habitat (Engel & Moran 2013; Pietri & Liang 2018).

Larvae from the resistant strain that had been fed with antibiotic food and had not been exposed to the insecticide (CoRe+Ab) had the lowest values of all treatments in larval growth rate, pupation rate, pupal weight, survival until adulthood and fecundity. This might have been a consequence of a presumed virus infection in the second batch that affected the boxes of three treatments (control exposition/ resistant strain/ standard diet, control exposition/ resistant strain/ antibiotic diet, and control exposition/ susceptible strain/ antibiotic diet), where some of the larvae died before pupation and the boxes had to be discarded before all larvae could pupate. But interestingly it's only the CoRe+Ab treatment that differs significantly from the other treatments. More replicates are needed to evaluate if the low performance of the CoRe+Ab treatment might have been an artifact due to the presumed virus infection or if the combination of cost of resistance and damaged gut microbiota in this treatment was especially costly in the absence of insecticides.

4.4. Implications for pest management

While survival of insecticide exposition in my study was found to be enhanced for *S. littoralis* larvae that had been treated with antibiotics prior to insecticide exposition, there are contrasting result from other studies where antibiotic treatment prior to insecticide exposition increased insecticide susceptibility in lepidopteran species. This was for example shown for *Bombyx mori* (Chen et al. 2020) as well as for *Spodoptera litura* (Gadad & Vastrad 2016). Thus, the effect of gut microbiota on insecticide efficiency seems to be difficult to generalize as it might vary depending on insect species (Pietri & Liang 2018; Phalnikar et al. 2019), bacterial strain (Xia et al. 2018), type and quantity of insecticides and antibiotics used (Frankenhuyzen et al. 2010; Hilbeck et al. 2018; Barnard et al. 2019) and environmental circumstances. And even the composition and abundance of bacteria in the gut microbiota is extremely variable and influenced by many factors such as diet, immune status, development and gut characteristics (Engel & Moran 2013). As potential consequences of gut microbiota manipulation on insecticide efficiency are hard to predict and antibiotic

treatment has been shown to enhance survival of insecticide exposition in my study and other previous research (Broderick et al. 2009; Visweshwar et al. 2015; Hilbeck et al. 2018; Barnard et al. 2019), caution might be necessary when manipulation of gut microbiota, for example with antibiotics, is suggested as a solution to insecticide resistance development. Despite the apparent complexity in the effect of host-microbial symbioses on insecticide resistance, there is growing interest to target insect-microbial symbioses for pest management purposes in agriculture. There have been suggestions to impair development and survival of insects through the use of antibiotics or through genetic manipulation of the genome of insect-associated bacteria (Berasategui et al. 2016; Arora & Douglas 2017). Knowledge about gut symbionts might also be used to improve biological pest control with parasitoids or sterile insect techniques (Berasategui et al. 2016). However, these methods have mostly been tested in highly controlled environments and several technical and severe ethical problems currently prevent a potential commercialization and large scale use of such pest management strategies that in the field (Berasategui et al. 2016; Arora & Douglas 2017). But potential solutions to these technical and ethical limitations are underway that might enable production of gut symbionts that are currently not culturable (Berasategui et al. 2016; Xie et al. 2019) and improve specificity of the microbial agents and limit their activity radius (Arora & Douglas 2017).

Costs of insecticide resistance development such as reductions in growth and fecundity that I found for resistant *S. littoralis* in my study seem to be a fitness disadvantage in the absence of insecticide exposition compared to susceptible individuals. Pest management can benefit from such costs of resistance for example by employing the refuge strategy in which areas free from insecticide selection pressure are established in order to delay the spread of insecticide resistance within an insect population (Abbas et al. 2012; Carrière et al. 2012; Kliot & Ghanim 2012).

The positive effect of antibiotic treatment on survival of insecticide exposition observed in my study might present implications for the insecticide efficiency in the field as there is a risk that antibiotics might be part of lepidopteran diet even in the field. Streptomycin and Amipicillin, the two antibiotics used in this study are widely used in agriculture globally (Manyi-Loh et al. 2018). Overuse of antibiotics, especially in livestock farming (e.g. as growth promoters), causes antibiotic pollution of ecosystems and insect habitats (Manyi-Loh et al. 2018). In this way irrigation water might get contaminated with antibiotic residues which might be consumed by pest insects feeding on the irrigated plants. If a field is then treated with insecticides, the combination of antibiotic diet and insecticide

exposition might be partly resembling the conditions in my study and might reduce insecticide efficiency. Even if insects do not get into contact with antibiotics in the field, other pesticides that are routinely used in agriculture might have similar effects. The widely used herbicide glyphosate for example is known to have antibacterial effects and to disturb insect gut microbiota (Gill et al. 2018; Motta et al. 2018; Van Bruggen et al. 2018; Gómez-Gallego et al. 2020). Such a disturbance in the gut microbial community might affect insecticide efficiency as well. Moreover, there might even be a connection between excessive use of insecticides and gut symbiont-mediated insecticide resistance. Increased pollution with an insecticide in the environment, might lead to accelerated biodegradation of the insecticide in the soil and increase the amount of soil bacteria that can break down this specific insecticide (Kikuchi et al. 2012). Through infections with these soil bacteria, insects might instantly gain insecticide resistance which might even be transferred horizontally between insects (Kikuchi et al. 2012). Since Cypermethrin can be found frequently in the environment (Tang et al. 2018), pyrethroids are often degraded by soil microorganisms (Chen & Zhan 2019) and free-living *S.littoralis* usually spend parts of their life time on and in the soil (Gawaad & El-Gayar 1974), future research could study if Cypermethrin-resistance might be transferred horizontally between *S. littoralis* in the field.

However, even if increased knowledge about the effect of insect gut microbiota on insecticide resistance development might aid in refining pest management strategies and even if new pest management methods targeting insect-microbial interactions and microbial-mediated traits would be successful, evolutionary changes in the insect host, its gut microbiota or their symbiosis might reduce efficiency of these pest management methods. *Any* pest control strategy selects for resistance due to evolutionary mechanisms (Arora & Douglas 2017; Karlsson Green et al. 2020). Therefore, it is essential to use insecticides only if there is absolutely no other alternative and to use integrated pest management strategies with diverse control measures and much focus on proactive, preventive measures to control pest insects and resistance development (Karlsson Green et al. 2020). Apart from the issues connected to resistance development, insecticides should also be used as little as possible due to their negative impact on natural ecosystems and human health (Koureas et al. 2012; Ansari et al. 2014; Carvalho 2017; Chen & Zhan 2019; Le Goff & Giraudo 2019). Therefore, it is important to implement more sustainable pest control measures and adopt agricultural practices that use preventive methods such as increasing biodiversity to attract natural enemies to reduce insect pest pressure. Higher agricultural biodiversity might not only facilitate

beneficial insects and their gut microbiome, but might also increase production and pollination and reduce pest and pathogen pressure (Dahlin et al. 2018; Yang et al. 2019; Zytynska & Meyer 2019; Tamburini et al. 2020).

4.5. Future research and further questions

The extent of relevance of gut microbiota for lepidopteran species has been controversial. My study seems to fit with other research done on *S. littoralis* showing that gut microbiota seemed to facilitate nutrition uptake (Chen et al. 2016), but also seemed to create costs since survival of insecticide exposition was increased when gut microbiota was damaged. However, as the effect of the gut microbiota on *S. littoralis* survival, development and fecundity seems to be quite diverse depending on environmental circumstances and gut bacteria involved, future research might sequence the composition and diversity of *S. littoralis* gut bacterial community more precisely to identify individual bacterial taxa and their function for the host. Dilution and plating technique have the disadvantage that only a small fraction of the insect gut bacteria can be cultured on laboratory media and that the cell count is quite unprecise (Pepper & Gerba 2015). Furthermore, since several *S. littoralis* guts were mixed in my samples, interindividual gut microbial differences within the same population could not be investigated which might be of interest for future research.

In the insect rearing at the laboratory *S. littoralis* eggs were routinely disinfected with formaldehyde fumes for 30 minutes before being used for experiments. This sanitary measure might affect the larval gut microbiota, because some major taxa associated with the female *S. littoralis* gut microbiota might be vertically transmitted to the next generation on the eggs (Chen et al. 2016). It would be interesting to compare the composition and function of the gut microbiota of *S. littoralis* larvae hatched from formaldehyde-treated and untreated eggs to evaluate the confounding effect of this sanitary routine for research results.

Field-collected and laboratory-reared larvae might differ greatly in composition and function of their gut microbial community (Staudacher et al. 2016; Gomes et al. 2020). While I only tested the effect of one specific insecticide (Cypermethrin), insects in the field are often exposed to several pesticides simultaneously and also to environmental stressors that are lacking in the laboratory (e.g. host plant toxins, pathogens and natural enemies) and these additional factors might affect the relationship between gut microbiota and insecticide resistance. Potential fitness costs of insecticide resistance might therefore be missed under laboratory

conditions (Kliot & Ghanim 2012). My experiments could be replicated with field-collected larvae and with more strains with greater genetic variation to test if the results found in my study can be generalized to other *S. littoralis* strains in other environments. Another important area for future research is variation of diet. The larvae in my study were fed with artificial diet, which might differ greatly from the polyphagous diet of wild *S. littoralis* populations. Since diet is assumed to have a major influence on composition of gut microbiota (Douglas 2015) and there have been hints that fitness costs are significantly larger for insects feeding on plants than on artificial diets (Gassmann et al. 2009), it might be interesting to study the mediating effect of different diets on the relationship between *S. littoralis* gut microbiota and insecticide resistance.

5. Conclusion

Increasing knowledge about the effect of gut microbiota on insecticide resistance development is crucial for refining pest management strategies which is needed to ensure global food security and to tackle environmental problems in agriculture.

Antibiotic treatment with Ampicillin and Streptomycin prior to insecticide exposition reduced susceptibility to Cypermethrin in a resistant and a susceptible *S. littoralis* lab strain in my study. It might be concluded that gut microbiota of the *S. littoralis* larvae in my study did not harbor any Cypermethrin-detoxifying bacteria. However, intact gut microbiota might have harbored opportunistic gut bacteria that became pathogenic and reduced survival following Cypermethrin exposure. Insecticide exposure reduced larval survival but increased larval growth rate, pupal weight, and fecundity. Thus, there might be long-term consequences of insecticide exposure on life-history traits of insects. Development of resistance to the insecticide Cypermethrin seemed to generate fitness costs in the resistant *S. littoralis* lab strain. The resistant strain had shorter larval and pupal development time and increased pupation rate, but lower larval growth rate, pupal weight, fecundity, and survival until adulthood compared to the susceptible strain. Gut microbiota seemed to mediate the costs of resistance as well as the consequences of insecticide exposition, even if no Cypermethrin-detoxifying effect of *S. littoralis* gut microbiota could be observed. Therefore, the mediating influence of gut microbiota is an interesting factor that should be considered in future research about insecticide resistance and the costs and consequences of insecticide exposure.

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Appendix 1 – Recipe for the standard *S. littoralis* diet

Spodoptera Diet

Wheat-germ	400 g	Yeast-mixture
Dried yeast Flakes	240 g	
Methyl-1-4-hydroxybenzoate	20 g	
Sorbic acid	20 g	
Ascorbic acid	22 g	
Cholesterol	8 g	
Distilled water	3.6 l	
Peeled potatoes	1700 g	
DL-α-Tocopherol acetate;		
Vit E (in fridge)	4 ml	
Oil	10 ml	
96% Ethanol	100 ml	
Plant agar (powder)	65 g	
Vitamin-mixture (in fridge)	24 g	
Sodium benzoate	6 g	

- Put the water to boil on the stove
- Peel the potatoes
- Slice and mash the potatoes in a mixer
- Add oil, Vit E and ethanol → Stir
- Add the yeast mixture → Stir
- Whip the Agar powder into the boiling water

- ❖ Pour the Water-agar mixture into the potato-yeast mixture little by little, **while stirring**
- ❖ When the mixture reaches 50-60 °C add the vitamin-mixture and the sodium benzoate → Stir well
- ❖ Pour hot mixture into the containers for storage and let them cool.
- ❖ Put the containers into the freezer.

Vitamin Mixture recipe

Eight ingredient mixture:

- 10g Nicotinamide (Sigma 72340, CAS 98-92-0)
- 10g D-Pantothenic acid calcium salt (Sigma 21210, CAS 137-08-6)
- 5g Riboflavin, Vitamin B2 (Sigma R4500, CAS 83-88-5)
- 2,5g Thiamine, Vitamin B1(Sigma T4625, CAS 67-03-8)
- 2,5G Pyridoxolhydrochlorid, Vitamin B6-hydrochlorid (ICN 101725, CAS 58-56-0)
- 2,5g Folic acid (CAS 59-30-3)
- 0,2g D-Biotin, Vitamin H (CAS 22879-79-4)
- 0,002g Cyanocobalanin, Vitamin B12 (Sigma V2876, CAS 68-19-9)

➢ Mix 2g of above stock mixture with 80g of ascorbic acid → **vitamin-mixture.**

Appendix 2 – Pilot study

The pupose of the pilot study was to develop a method for manipulation of the gut microbiota of *Spodoptera littoralis* larvae in order to assess if gut microbiota had any influence on survival of insecticide exposition or performance in different life-history traits. In order to manipulate *S. littoralis* larval gut microbiota, antibiotics were added to the *S. littoralis* diet.

The aim of the pilot study was to test if adding antibiotic solution to larval food can affect larval gut microbiota and to find an effective combination of different antibiotics to use as antibiotic cocktail that can be added to the food. Another important research question was if larval survival would be negatively impacted through the addition of antibiotics to the larval diet.

The pilot study was performed twice, first in larvae that were reared individually and then in larvae that were reared in groups as the first study indicated that individually reared larvae had little gut microbiota at all.

To find suitable combinations and doses of antibiotics that could be used to manipulate gut microbiota, inspiration was taken from previous research (Lin et al. 2015; Visweshwar et al. 2015; Thakur et al. 2016; Bai et al. 2019; Phalnikar et al. 2019; Ravenscraft et al. 2019) and four different antibiotic solutions were prepared (Table 12). The different antibiotic ingredients were weighed and mixed with MilliQ water in a Falcon tube. Besides these 4 antibiotic solutions, autoclaved MilliQ water was used as control.

Table 12. Combinations and doses of antibiotics used in the different treatments of the pilot study

Antibiotic solution	**Quantity of antibiotic per 1 ml H_2O**
tetracycline, ampicillin and streptomycin combined	0.12 mg tetracycline 0.4 mg ampicillin 0.4 mg streptomycin ---------- = 0.92 mg antibiotic
ampicillin and streptomycin	0.4 mg ampicillin 0.4 mg streptomycin ---------- = 0.80 mg antibiotic
streptomycine	3 mg streptomycin
ampicillin	3 mg ampicillin

Pilot study 1 – manipulating the gut microbiota of individually-reared *S. littoralis* larvae

50 µl of either one of four antibiotic solutions (Table 12) or the control (autoclaved MilliQ water) were pipetted onto 150 mg of standard *Spodoptera littoralis* food (Appendix 1). Each treatment group (4 different antibiotic treatments and a control treatment) had 40 larvae that were kept and fed individually in small petri dishes (12 cm circumference, 1 cm high, 3.5 cm diameter) with lid. All larvae in the different treatments were from the same laboratory strain. The larvae were kept and fed individually in the petri dishes for one week and survival of the larvae was recorded. Every other day new 150 mg pieces of *S. littoralis* food were drizzled with either an antibiotic solution or control (according to the treatment) and placed in the petri dishes.

After one week four larvae were chosen randomly from each of the five treatments (the four different antibiotic treatments and the control) and dissected in order to see if the antibiotic food affected the gut microbiota of the *S. littoralis* larvae and if the gut microbiota could be manipulated through antibiotic food.

The larvae that had been selected were starved two hours prior to dissection. The larvae were then put in cups and stunned on ice for a while. Directly before gut extraction, that was performed in sterile conditions under a lamina hood, each larva was first dipped into a beaker with 95% ethanol and then into a beaker with autoclaved Milli-Q water. The larvae were dissected using two forceps that were sterilized with 95% ethanol between dissection of the different larvae.

For each treatment and replicate, four larval guts were extracted and placed into an 1.5 ml Eppendorf tube filled with 1ml autoclaved Milli-Q water. The Eppendorf tube was vortexed for approximately 30 seconds and then 100 µl from the solution was transferred to another 1.5 ml Eppendorf tube filled with 900 µl autoclaved Milli-Q water. A dilution series was performed, transferring $1/10^{th}$ of the solution as many times as was necessary in order to reduce the number of bacterial colonies on the agar to make counting by eye feasible. 100 µl from each of the different dilutions were pipetted and spread on petri dishes with LB Agar (500 ml milliQ water + 20 g LB Agar). The petri dishes with the diluted larval gut solution were incubated for 72 hours at 30°C in an incubator. After that bacterial colonies were counted on the plates and the number of CFU per ml gut solution was calculated.

The above describes experiment was replicated with larvae from the same batch first one day later and then even four days later.

RESULTS from Pilot study 1

Survival of the larvae in the different treatments was recorded over one week until dissection to see potential influence of the different diets on survival (Table 13). Survival rate for the larvae in the different treatments was calculated pooled over the two replicates (2x20 larvae per treatment).

Table 13. Survival rate of larvae in Pilot study 1 (number of larvae in each treatment was 40, pooled from 2 replicates with 20 larvae per treatment)

Treatment	Survival rate after 7 days
Control	90%
Ampicillin, Streptomycin & Tetracycline	92.5%
Ampicillin & Streptomycin	87.5%
Streptomycin	95%
Ampicillin	85%

There were no substantial differences between survival rates of the larvae in the different treatments.

Results from bacterial count after dissection of seven-day-old larvae showed that after 72 hours of incubation not a single bacterial colony was growing on any of the petri dishes, neither on one of the antibiotic treatments nor on one of the control treatments.

Results from the replicate of larvae from the same batch that were four days older showed a difference of number of bacterial colonies per ml of gut solution between the antibiotic treatments and the control (Table 14).

Table 14. Amount of colony forming units (CFU) per ml of gut solution dissected from 11-day old larvae from the different treatments

Treatment	CFU/ml
Control	9420
Ampicillin, Streptomycin & Tetracycline	0
Ampicillin & Streptomycin	0
Streptomycin	0
Ampicillin	0

No bacterial colonies were found on the petri dishes that were prepared from guts extracted from *S. littoralis* larvae that had been fed with antibiotic food. Petri dishes prepared from larvae that had been fed with standard food without antibiotics on the contrary had grown bacterial colonies. These results imply that the antibiotic diet changed the larval gut microbiota and thus might be used as a method to manipulate gut microbiota in *S. littoralis* larvae.

Pilot study 2 – manipulating the gut microbiota of *S. littoralis* larvae reared in groups

Since no colonies grew on the petri dishes prepared from the guts of individually reared seven-day-old larvae in pilot study 1, the larvae in pilot study 2 were reared and fed in groups of 40. Larvae from the same treatment were reared together in one box to see if larval density might affect gut microbiota and the quantity of bacterial colonies on petri dishes after gut dissection. 500 µl antibiotic solution or autoclaved MilliQ-water (control treatment) was pipetted on 1.5 g of food. Four 1.5 g pieces of treated food were placed in each box with 40 larvae 2-3 times per week. Larval survival was monitored, and gut dissection, petri dish preparation and incubation were performed in the same way as described in pilot study 1.

RESULTS from Pilot study 2

Survival of the larvae after five days was worst in the control treatment (Table 15). Possibly some disease might have entered the box with the larvae from the control treatment that did not enter the other boxes or that the antibiotic food helped the larvae from the other treatments to survive. Unfortunately, all larvae from the same treatment were reared in the same box so that it is not possible to exclude confounding box effects.

Table 15. Survival rate of larvae reared in groups for the different treatments after 5 days

Treatment	Survival rate after 5 days
Control	35%
Ampicillin, Streptomycin & Tetracycline	67.5%
Ampicillin & Streptomycin	97.5%
Streptomycin	90%
Ampicillin	100%

Results from the dissection showed that there was a large difference in number of colony forming units (CFU) growing from the gut solutions extracted from larvae from the different treatments (Table 16).

Table 16. Number of colony forming units (CFU) per ml of gut solution dissected from seven-day-old group-reared larvae from the different treatments (results are mean value of 3 replicates)

Treatment	CFU/ml
Control	1486667
Ampicillin, Streptomycin & Tetracycline	7
Ampicillin & Streptomycin	0
Streptomycin	10
Ampicillin	0

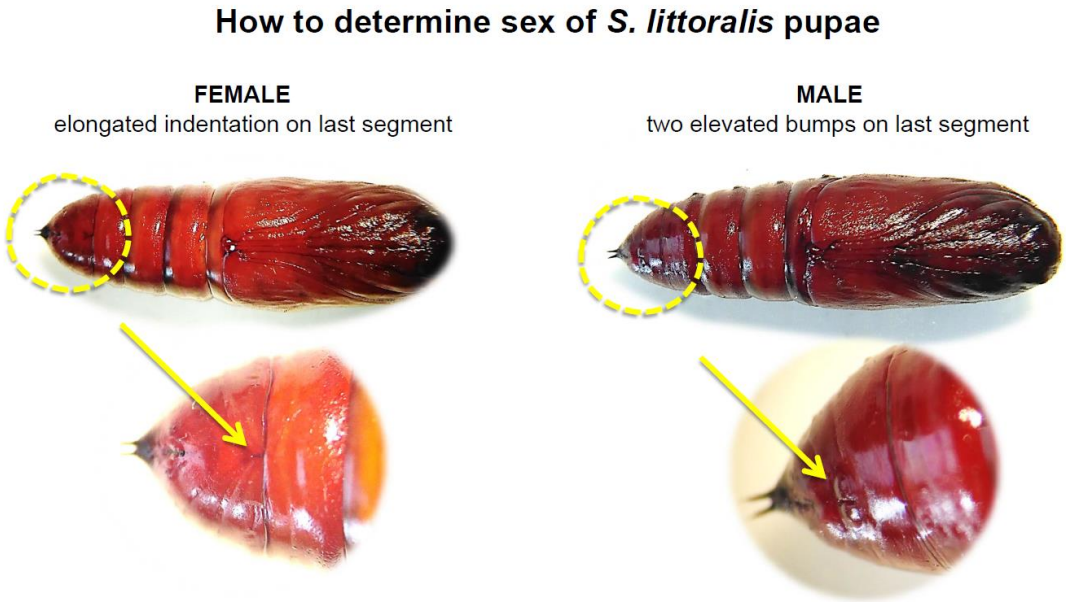
Summarizing the results from pilot study 1 and 2 (Table 17) it was concluded that it seemed possible to manipulate gut microbiota with antibiotic food and that this method seemed sufficient for the experiments in the Master thesis described in this paper. It was reasoned that an antibiotic cocktail consisting of several antibiotics would be more sufficient and would work broader in manipulating gut microbiota than a single antibiotic. Therefore, the antibiotic cocktail including Ampicillin and Streptomycin was chosen for the experiments, since it showed a little higher survival rate and little lower number of CFU/ml than the other antibiotic cocktail tested.

Table 17. Comparison of survival rate and number of CFU per ml gut solution over the different treatments and individually vs group reared

Treatment	**Survival rate in %**		**CFU/ml**	
	individually-reared	group-reared	individually-reared	group-reared
Control	90	35	9420	1 486 667
Ampicillin, Streptomycin & Tetracycline	92.5	67.5	0	7
Ampicillin & Streptomycin	87.5	97.5	0	0
Streptomycin	95	90	0	10
Ampicillin	85	100	0	0

Appendix 3 - How to sex *Spodoptera littoralis pupae*

Sexual dimorphism on the last segment of the pupal body was used to define the sex of *S. littoralis* pupae. Female pupae have an elongated indentation on the last segment and male pupae have two elevated bumps on the last segment.



Appendix 4 Result tables

Survival following exposition with either Cypermethrin or control

Table 18. Survival after exposition experiments. At the outstart of the exposition experiment 100 larvae were included in each treatment (50 in each replicate). The table shows the total number of individuals that were still alive after 72 hours of exposition with either Cypermethrin or a control.

Strain	Diet	Exposition	Number of individuals alive after 72 hours of exposition		
			Batch A	Batch B	Both batches together
susceptible	standard	Cypermethrin	17	7	24
susceptible	standard	control	50	50	100
susceptible	antibiotic	Cypermethrin	27	9	36
susceptible	antibiotic	control	50	50	100
resistant	standard	Cypermethrin	36	19	55
resistant	standard	control	50	50	100
resistant	antibiotic	Cypermethrin	50	38	88
resistant	antibiotic	control	50	50	100

Eclosion rate for male and female *S. littoralis* pupae

Table 19 Number of pupae, adults and eclosion rate of male and female S. littoralis for the different treatments

Strain	Diet	Exposition	Number of pupae		Number of eclosed adults		Eclosion rate (adults/pupae)	
			♀	♂	♀	♂	♀	♂
susceptible	standard	Cypermethrin	10	10	9	8	0.90	0.80
susceptible	standard	control	45	41	39	32	0.87	0.78
susceptible	antibiotic	Cypermethrin	14	16	14	14	1.00	0.88
susceptible	antibiotic	control	41	46	39	44	0.95	0.96
resistant	standard	Cypermethrin	23	24	22	18	0.96	0.75
resistant	standard	control	36	27	33	26	0.92	0.96
resistant	antibiotic	Cypermethrin	37	43	35	41	0.95	0.95
resistant	antibiotic	control	25	24	23	22	0.92	0.92