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Franziska Dickel

Medicative Diet – the Importance of Diet and Prophylactic Treatment on Survival and Immunity of Polyphagous *Arctia plantaginis* (Arctiidae) larvae

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“ If we knew what we are doing it wouldn’t be called research, would it?”
Albert Einstein

“Lass dich nicht unterkriegen, sei frech und wild und wunderbar”
Astrid Lindgren

ABSTRACT

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Yhteenveto: Ruoan ennaltaehkäisevä vaikutus polyfaagin perhosen *Arctia plantaginis* (Arctiidae) elinkykyyn ja immuniteettiin
Diss.

Diet is one of the major factors directly and indirectly influencing insect's life history traits and risk of getting infected. Additionally the insect's fitness is severely affected by the broad diversity of parasites they are exposed to. As a consequence insects have developed well-evolved defences. Behavioural responses include self-medication, the ability of insects to change dietary intake in response to an infection. When studying this ability it is of major importance to consider the insects natural diet range. In this thesis I investigated the effect of different host plants on fitness and immunocompetence of polyphagous *Arctia plantaginis* larvae and whether the larvae can therapeutically and prophylactically self-medicate by optimising their diet intake. Additionally I examined the long-term effect of prophylactic treatment on lab-reared larvae from the same species. Feeding experiments reveal that the host plants plantain (high in biologically active compounds) and dandelion (high nutritional value) have different effect on the larval development and survival. Results show that a monotonous plantain diet provides lowest protection against an infection, whereas a diet switch from plantain to dandelion increases survival. Immunocompetence seems to be not differently affected by the two host plants. When given the choice, all larvae choose to ingest a mixture of both plants. The ratio of both plants differed depending on the infection status or infection risk in their environment compared to control larvae. This highlights the importance of a mixed diet for some polyphagous species and that dietary variety should be considered when studying insects' ability to therapeutically and prophylactically medicate. A prophylactic medication applied to laboratory-reared insects showed negative effects on life-history traits and reproductive success, and thus should be carefully considered. In conclusion these findings provide insights in the crucial importance of diet mixing for polyphagous larvae and show evidence for the larvae ability to therapeutically and prophylactically medicate by mixing their diet.

Keywords: *A. plantaginis*; diet-mixing; immunity; medication; prophylaxis.

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CONTENT

LIST OF ORIGINAL PUBLICATIONS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

I Dickel, F., Mappes, J. & Freitak, D. 2017. Variation in diet can increase the resistance of polyphagous larvae against an infection. Manuscript.

II Dickel, F., Freitak, D., Stucki, D. & Mappes, J. 2017. Polyphagous larvae know how to stay healthy. Manuscript.

III Dickel, F., Murphy L., Freitak, D. & Mappes, J. 2017. Prophylaxis under high infection risk: Can polyphagous larvae avoid getting sick? Manuscript.

IV Dickel, F., Freitak, D. & Mappes, J. 2016. Long-term prophylactic antibiotic treatment: effects on survival, immunocompetence and reproduction success of *Parasemia plantaginis* (Arctiinae). *Journal of Insect Science* 16: 46.

The table shows authors contributions to original papers. Smaller contributions are stated in the acknowledgements of the original papers.

	I	II	III	IV
Original idea	FD, JM, DF	FD, DF, JM	FD, JM	JM
Data collection	FD, JM, DF	FD, DF	FD, LM	FD, JM
Statistics	FD	DS, FD	LM, FD	FD
Manuscript	FD, DF, JM	FD, JM, DF, DS	FD, LM, JM, DF	FD, JM, DF

DF = Dalial Freitak, DS = Dimitri Stucki, FD = Franziska Dickel, JM = Johanna Mappes, LM = Liam Murphy

1 INTRODUCTION

1.1 Diet

Nutrition is of key importance for all organisms. A balanced diet of nutrients, minerals and vitamins is necessary to maintain growth, reproduction and energy uptake and can thus probably strongly affect organisms fitness (Chapman 2013). This effect has been proven for insects by many scientific studies (Ojala *et al.* 2005, Alaux *et al.* 2010). From these studies we know that diet can directly influence insects growth, development, reproduction, lifespan and also the immune response (Fox *et al.* 1990, Awmack and Leather 2002, Lill *et al.* 2002, Ojala *et al.* 2005, Zhang *et al.* 2012, Chapman 2013). Diet affects these factors by prolonging, increasing or stopping them. Immune response can be suppressed in response to an unbalanced diet intake (Lee *et al.* 2008). Diet can also indirectly increase the risk of predation for the insect, or the probability of infection and parasitism (Fox *et al.* 1990, Lill *et al.* 2002). It is not only the food availability but also the food quality and food choice that affect these above-mentioned changes of insects' fitness. Diet quality can affect the insects' susceptibility for infections and disease (Cory and Hoover 2006; Zhang *et al.* 2012; Singer *et al.* 2014). Short term food deprivation results in a reduced immune response in the mealworm beetle *Tenebrio molitor* (Siva-Jothy and Thompson 2002). However, there are also positive effects documented, a protein-rich diet inreases the immunocompetence of *Apis mellifera* and caterpillars of *Spodoptera littoralis* (Lee *et al.* 2008, Alaux *et al.* 2010), bumble bees show a reduced pathogen load of *Crithidia bombi* when feeding on a gelsemine rich diet (Manson *et al.* 2010), and Lepidoptera larvae show higher encapsulation ability when feeding on a diet high in antioxidants (Ojala *et al.* 2005). Dietary intake clearly affects the insects' ability to survive an infection by manipulating their defence mechanisms against non-self. Additionally, from the diet itself, microorganisms on the food surface or for example within plants can also affect them.

1.2 Insects anti-parasite defence mechanisms

1.2.1 Overview

Parasite pressure is one of the greatest challenges insects are exposed to during their development (Lefèvre *et al.* 2010, Schmid-Hempel 2011, de Roode and Lefèvre 2012, Mikonranta *et al.* 2017). In addition, herbivorous insects encounter a great variety of plant allelochemicals within their diet. In response to the threat of parasites and chemicals, insects have evolved a great variety of defence mechanisms to protect themselves and have adapted to chemicals in their diet. They have evolved sequestration and detoxification mechanisms for secondary metabolites to use them for their own defence (Christe *et al.* 2003, Hartmann *et al.* 2005, Harvey *et al.* 2005, Baden and Dobler 2009, Singer *et al.* 2009, Zhang *et al.* 2012)

1.2.2 Morphological defences

Morphological defences consist of physical and chemical barriers, which the pathogen needs to overcome as a first defence line by the insect. Physical barriers are the insects' cuticle, hairs or spines, which decrease the parasites ability to penetrate the host (Schmid-Hempel 2005, 2011), or colouration which protects them against predators (Gross 1993, Lindstedt *et al.* 2008). A chemical barrier for example, is the application of antibiotics to the insects body. This antibiotics can be used as defence against fungi, and is a commonly used defence mechanism by ants. (Ortius-Lechner *et al.* 2000). Also the gut endothelia is part of these morphological defences (Paul and Ebert 2003, Schmid-Hempel 2011).

1.2.3 Insect immunity

Once parasites have overcome the pre-infection mechanisms of insects they then face the post-infection defences. Apart from grooming behaviour and behavioural fever (Hart and Ratnieks 2001), the main physiological post infection defence is the insects immune system (Schmid-Hempel 2011). Insect immune system is a well-evolved defence against infections, which gets initiated once the insect has recognised the infection. This occurs via special recognition proteins, which recognise special characteristics of the microbes surface (pathogen-associated molecular patterns, PAMPs). By this the insect can distinguish non-self from self (Schmid-Hempel 2005) and activates the defence response.

The insects' immune system is composed of humoral and cellular responses. (Gillespie *et al.* 1997, Vilmos and Kurucz 1998, Strand 2008a, Singer *et al.* 2014). The humoral responses are based on non-cellular components and the production of antimicrobial peptides (AMPs). After recognition of an infection these AMPs are produced in the fat body and haemocytes where they fight the infection. Additionally, they are also produced in the gut ephitelia (Lemaitre *et al.* 2007, Haine *et al.* 2008, Rolff and Reynolds 2009). One example of AMPs is lysozyme, which is produced in the fat body and haemolymph. Its production is up regulated in response to recognition of bacteria. The enzyme degrades the bacterial cell wall (Tsakas and Marmaras 2010). The cellular response is based on a variety of different specialised cells, the prohaemocytes and haemocytes (Hartenstein 2006, Schmid-Hempel 2011). Mechanisms of cellular response include phagocytosis, encapsulation, and the activation of prophenoloxidase (proPO) (Strand 2008b). Phagocytosis is performed by special haemocytes, detecting and eliminating pathogens. The phagocytic haemocytes aggregate around the bacteria and destroy them (Gillespie *et al.* 1997, Schmid-Hempel 2005, Rosales 2011). During this process the invasive organism or cells might be also killed by reactive oxygen species (ROS, super oxides, hydrogen peroxidase), which are produced by the insect in an oxidative burst. Oxygen species can cause oxidative stress and cell damage for the insect itself but also play an important role in intracellular signalling and insect innate immune defence (Schmid-Hempel 2011, Zug and Hammerstein 2015). ProPO is activated by special PAMPs from microbial cells and tissue damage. Its inactive form then converts to phenoloxidase (PO), producing phenols, quinones and ROS, which are directly active against parasites, viruses, bacteria and fungi. Additionally, the activation process also leads to the polymerisation of melanin, which deposits around foreign objects (Cerenius and Söderhäll 2004, Cerenius *et al.* 2008, Lee *et al.* 2008, Schmid-Hempel 2011, González-Santoyo and Córdoba-Aguilar 2012).

The up-regulation of the immune system is costly and requires energy, which can be gained by nutrients. Diet quality and quantity thus affect the immune response of insects (Siva-Jothy and Thompson 2002, Schmid-Hempel 2003, Lee *et al.* 2008). Development and immune response both require nutrients but can be influenced in opposing ways by the same compound. Lysozyme-like activity and PO activity for example require high amounts of amino-acids, while they limit insects growth (Schoonhoven *et al.* 2005, Singer *et al.* 2014b), and proteins can suppress immune response while they are highly essential for insects development (Lee *et al.* 2006a, Povey *et al.* 2009, Raubenheimer and Simpson 2009). Furthermore, ingested secondary metabolites can directly affect immune responses by impairing metabolic processes, as shown by Smilanich et al. (2009).

1.2.4 Behavioural defences

Behavioural defences are used before and after infection by insects. Pre-infection behavioural defences mostly include the avoidance of getting infected, repelling the parasite or a change of behaviour or group life (Schmid-Hempel 2011, de Roode and Lefèvre 2012). In social insects, hygiene and grooming are common forms of behavioural avoidance. Honeybees, ants and termites remove infected individuals from the hive to avoid spread of the disease, beetles and termites groom themselves or nest-mates bodies with their leg to remove parasites or fungi, and social insects store food and waste products in separate areas within the nest (Rothenbuhler 1964, Gaugler *et al.* 1994, Hart and Ratnieks 2001, Weiss 2006, Yanagawa and Shimizu 2007, Wilson-Rich *et al.* 2009). Some insects spatially or temporally avoid getting infected by changing their place of oviposition or foraging time (Orr 1992, Amano *et al.* 2008). Additionally compensatory consumption and enhanced food utilisation may represent a form of behavioural defences, which are strongly reliable of the diet composition (Schoonhoven *et al.* 2005).

1.3 Medication

1.3.1 Overview

Animal medication is a main form of behavioural defence (Singer *et al.* 2014). It is known in invertebrates and vertebrates (de Roode and Lefèvre 2012). Some diet compounds can influence the pathogens effect on animals. Secondary metabolites negatively affect an insect, as they are produced as a defence against herbivores by plants. However, they may also affect pathogens to the same extent, which will result in a positive effect for the host insect (Price *et al.* 1980, Cory and Hoover 2006). Adding the secondary metabolite gelsemine to bees diet, resulted in reduced pathogen load of *Crithidia bombi* (Manson *et al.* 2010). Monarch butterflies that feed on milkweed show reduced parasite growth (De Roode *et al.* 2008). When these substances are consumed actively, the behaviour is referred to as self-medication. It is defined as a specific behavioural change, therapeutically or adaptive, in response to a disease (Abbott 2014, Singer et al. 2014b). There are four criteria that must be met to call this change in behaviour as true self-medication. Abbott (2014) defines them as following: I) deliberate contact or consumption of the compound, II) harmful effect of the compound to the parasite or disease, III) contact or ingestion of the compound has a beneficial effect on host fitness, IV) if not infected, the host fitness is negatively affected by the compound. Importantly, medication behaviour is distinguished between prophylaxis and therapeutic medication (Abbott 2014, de Roode et al. 2013, Singer et al. 2009).

1.3.2 Therapeutic self-medication

Therapeutic self-medication is a change in feeding behaviour after an infection has occurred, resulting in an increased resistance against the parasite (Lozano 1998, Singer et al. 2014b). The behaviour is predicted to have fitness costs if performed without infection, also describing a case of adaptive plasticity (Singer et al. 2009). Some of the best-studied examples of self-medication behaviour describe primates ingesting whole leaves in response to nematode infections (Wrangham 1995, Huffman and Hirata 2004, Fowler *et al.* 2007). It is also increasingly recognised that such behaviours are common in insects, providing strong protection against parasites and parasitoids (de Roode and Lefèvre 2012).

A case of true self-medication, fulfilling all four mentioned criteria, is shown by a study by Bos *et al.* (2015). *Formica fusca* ants infected by the fungus *Beauveria bassiana* actively consumed reactive oxygen species, which resulted in an improved survival rate. When ingested by non-infected individuals, the same diet decreased their survival compared to individuals feeding on a control diet (Bos *et al.* 2015).

1.3.3 Prophylactic self-medication

Prophylactic self-medication is defined as the active contact or ingestion of a toxic substance in response to a high infection risk posed by a pathogen or parasite, without the host yet being infected (de Roode *et al.* 2013, Singer *et al.* 2014b, Abbott 2014). It can further be divided to constitutive prophylaxis (Harvell 1990, Singer *et al.* 2014b), the response to a ubiquitous risk, and induced prophylaxis, which characterises the response after sensing a risk of an infection. There is evidence for insects engaging in prophylactic medication, which mostly occurs in social insects. Ants and bees collect resin or propolis to protect their nests against parasites (Christe *et al.* 2003, Chapuisat *et al.* 2007, Simone-Finstrom and Spivak 2010, 2012). Non-social insects, like beetles and lepidopteran caterpillars show a greater resistance against infection in response to high population densities (Barnes and Siva-Jothy 2000, Cotter *et al.* 2004). High population densities were predicted to act as a cue for high infection risk for insects, which is why the monitored increased resistance might present a form of prophylactic medication (Wilson and Reeson 1998). The monarch butterfly or Drosophila females show an altered oviposition preference when under a infection or parasitoid risk (De Roode *et al.* 2008, de Roode *et al.* 2013). However here the problem arises that these cases are actually not self- but rather kin- medication, or that they do not meet all four criteria for true self-medication mentioned above. According to Abbott (2014), the response or contacted substance needs to impose a negative effect or cost to the individuals. Otherwise the behaviour would be a case of prophylactic consumption rather than medication. The measurement of possible costs as well as experimental set-up of introducing a high infection risk is to our knowledge still the biggest issue when trying to study the ability of insects to prophylactically medicate. In addition insects might consume a certain diet not to prevent infection but because it was the closest food to reach or matched their actual host plant the most (Behmer 2009, Abbott 2014).

1.4 Insect herbivores

In general, insects have rather similar nutrient requirements. Nevertheless, depending on their food specialisation and adaptation, these requirements might vary (Chapman 2013). Herbivorous insects make up almost half of all insect species, which is why around 400.000 insects feed on plants (Schoonhoven *et al.* 2005). The extent to which herbivory occurs in insect groups differs. While Lepidoptera and Orthoptera are almost completely herbivorous, large numbers of Coleoptera, Hymenoptera and Diptera are also herbivorous species. Insect herbivores are generally grouped by their degree of specialisation. Insects that occur and feed on plant species from a single genus are characterised as monophagous, which many lepidopteran larvae and coleoptera are designated as. Oligophagoues insects, such as the white cabbage butterfly and the Colorado potato beetle, have the ability to feed on different genera from one plant family. Polyphagous insects are herbivorous insects that can feed on plant species from different plant families (Schoonhoven *et al.* 2005, Ali and Agrawal 2012). Polyphagous feeders face a very broad dietary spectrum, accepting many different plants. The green peach aphid for example can feed on more than 50 plant families (Schoonhoven *et al.* 2005). Also the larvae of arctiid moth Arctia plantaginis (*A. plantaginis*) belong to polyphagous feeders (Conner 2008), though it is estimated that only between 10 and 20% of insects feed on plants from more than 3 families (Bernays and Graham 1988). Monophagous and oligophagous insects are grouped together as specialists, and polyphagous insects are characterized as generalists (Schoonhoven *et al.* 2005, Ali and Agrawal 2012). Most herbivorous insects are specialists, which dominate most orders and are generally assumed to outcompete generalists feeders due to selective advantages (Mooney *et al.* 2012).

Host-plant specialisation dominates most insect orders, though it can greatly vary within insect groups and even insect populations (Schoonhoven *et al.* 2005). Even though a high degree of host-plant specialisation might imply a loss of using many different plants as food source, specialists benefit by being highly adapted to the plant’s specific defences against herbivores (Ali and Agrawal 2012). This interaction between plant defence and host dietary specialisation was for a long time studied without taking into account the pressure by natural enemies on herbivores (Bernays and Graham 1988, Mooney *et al.* 2012, Muller *et al.* 2015). The tri-trophic interaction (TTI) hypothesis views plant-herbivore interactions by consolidating the influence and interaction of all three factors, natural enemies, host plant quality and herbivore diet breadth (Mooney *et al.* 2012). Moody et al. (2012) discuss the coexistence of specialists and generalists in the background of this three-factor-interaction, providing further knowledge for understanding the evolution of insect diet breadth. The TTI hypothesis is based on three smaller hypotheses taking into account their cross-comparisons. The enemy free space theory states a higher impact of enemies on generalist compared to specialists (Bernays and Graham 1988). The Slow-Growth/High-Mortality hypothesis predicts that a low host-plant quality enhances the effects of natural enemies (Williams 1999). The physiological efficiency hypothesis predicts that generalists are more strongly affected by changes in host-plant quality (Dethier 1954) and predicts that specialists should be able to better escape predators and are dominant due to faster growth rates. This hypothesis is particularly relevant when the food quality is low. The main assumption is thus that specialist feeders outcompete generalists (Mooney *et al.* 2012).

Furthermore, specialist feeders are known to be well adapted to plants toxic defence components and it is noted that these chemicals can have beneficial effects on reducing disease symptoms in organisms (Schoonhoven *et al.* 2005). However, theory suggests that generalists may persist better in fluctuating, non-equilibrium environments. This might simply be based on their broader food range, as well as their ability to actually ingest and digest a broader variety of plant compounds, which they can adjust to faster than specialists. Furthermore, just the fact itself that some herbivorous insects still do have a generalist diet suggests that the maintenance of a diverse diet may confer certain fitness advantages that outweigh diet specialisation. A study on the polyphagous European grapevine moth showed that generalists show a good survival on rare alternative hosts, which also gives them the advantage of surviving in changing habitats (Thiery and Moreau 2005).

1.5 Plant compounds

Herbivorous insects are exposed to a variety of primary and secondary metabolites in their diet (Schoonhoven *et al.* 2005). Primary compounds make up essential nutrients for herbivores, like proteins, carbohydrates and lipids. Thus their quality but also quantity largely affects the insects feeding performance and development (Berenbaum 1995, Schoonhoven *et al.* 2005). Cellulose and lignin belong to this group of primary metabolites, although both are indigestible for insects. The quantitative variation of nutrients, high content of water and cellulose actually might not make plants the most suitable food source (Schoonhoven *et al.* 2005). Additionally, plants produce a great amount of different secondary metabolites, which do not have a special function in basic plant metabolism. They are non-nutritional chemicals involved in plants defence against insects and in the attraction of pollinators. Due to the toxic characters of some chemicals they are also called allelochemicals (Elsayed 2011). Apart from being toxic, secondary plant compounds can also be unpalatable for herbivores (Detzel and Wink 1993, Glendinning 2002). The main groups are nitrogen-containing compounds like alkaloids, terpenoids, phenolics and polyacetates (Schoonhoven *et al.* 2005, Elsayed 2011), with overall more than 100 000 compounds (Schwab 2003, Boege 2004). The quantity of secondary substances varies greatly between plant taxa but also between plant parts. The latter is based on the plants ability to allocate these compounds in different tissues, and this also protects the parts that are most likely to be attacked by herbivores (Van Dam *et al.* 1996, Schoonhoven *et al.* 2005).

1.6 Diet mixing

Feeding on plants exposes herbivores to a wide variety of different nutrient levels and secondary metabolites. As generalists can feed on a range of different plant species, they may actually gain best fitness when switching host plants to mix diets. This diet mixing would allow them to balance the quality and quantity of nutrients from different plants, and also allows them to dilute possible negative effects of allelochemicals (Bernays and Minkenberg 1997, Mody *et al.* 2007, Raubenheimer and Simpson 2009). Furthermore, some larvae need to change dietary intake during their development because instars require different nutrient composition. Hence, a diet switch might improved growth (Barbosa *et al.* 1986).

Studies on the effect of this kind of proposed diet mixing for generalist herbivore insects vary in their results. It is shown that it can indeed improve performance of several insect herbivores (Waldbauer, G P and Friedman *et al.* 1991, Hägele and Rowell-Rahier 1999, Miura and Ohsaki 2004). On the other hand a mixed diet can also imply negative effects for the insect or not differ compared to feeding on a single plant (Bernays and Minkenberg 1997). Bernay et al. studied the effect of a mixed diet with different species, measuring several parameters. Lepidopteran and hemipteran species did not show a clear beneficial effect when feeding on mixtures of host plants, but rather revealed a decreased performance (Bernays and Minkenberg 1997; Bernays 1999). While for gypsy moth a diet of different host plants did not show any different effect than a diet of one host plant (Stoyenoff *et al.* 1994), it is known that different host plants can be needed within the insects' development. A switch between host plants can then show a positive effect for the insect compared to continuous feeding on a single host plant during whole development (Barbosa *et al.* 1986) and some insects change their host plants even regularly during a day or switch to feed on different plant parts (Schoonhoven *et al.* 2005). Mody et al. 2007 showed an increased number of eggs laid by herbivore caterpillars feeding on a mixed diet, compared to individuals fed on a monotonous diet. This kind of diet mixing might have allowed a more optimal diet. Diet mixing thus seems to have different effect on generalist insect species. Apart from not clearly affecting them it can be important for polyphagous insects during normal circumstances and might become especially relevant after being exposed to environmental stressors (Waldbauer, G P and Friedman *et al.* 1991, Lee *et al.* 2006b). The nutrient requirement for healthy and infected individuals might greatly differ, for example due to the costly up-regulation of the immune system (Povey *et al.* 2009). Caterpillars consume greater amount of protein to carbohydrates in response to an infection (Lee *et al.* 2006), and show decreased growth rates when feeding on sub-optimal mixture of diet (Thompson and Redak 2005). A diverse diet during development can in general increase fitness of herbivore insects compared to a more monotonous diet (Mody et al., 2007).

Taking all this knowledge and evidence into account, it is of special importance to include the natural diet breadth of herbivorous generalists to studies assessing host-plant interactions. If a mixed diet is known to increase fitness of several insect species, then a diet switch might also actually provide them the best protection against an infection, and act as best medicine. Generalists may also perform self-medication behaviour by keeping up a mixed diet intake, rather then only switching to a pure intake of only one substance or diet.

1.7 Prophylactic treatment in laboratory reared insects

Studying medication behaviours is not only helping to understand insects' ability to adjust to environmental changes or shed light to insect-plant interactions. It might be also of great importance when mass-rearing insects under laboratory conditions. Insect rearing in big numbers is of great importance for pest management, production of chemical insecticides, food production for other animals and research purposes (Leppla 2009, Sørensen *et al.* 2012). Due to high population densities and artificial rearing conditions, the occurrence and spread of diseases and infections is a common problem in mass-reared insect populations. Prophylactic treatment is thus a commonly used method, such as adding antibiotics to the artificial diet. Similar to an active prophylactic food change when recognising a high infection risk, a certain substance is here applied to prime the population against possibly occurring infections. To our knowledge, it is not studied how the prophylactic treatment with antibiotics might affect populations in long-term. Even though negative effects might be suspected, there is no clear evidence showing a harmful effect on life-history traits of the insects. Wilkinson already raised this concern in his study in 1998, and highlighted developmental time and reproductive success as possible sensitive traits negatively affected by long-term prophylactic treatments (Wilkinson 1998).

1.8 Aims of the study

In this thesis I aim to investigate the different effect of two host plants on larvae of the polyphagous moth *A. plantaginis*, and the importance of a diet switch to gain best protection against infections but also highest fitness. Furthermore, I am aiming to show new evidence for medication behaviour by mixing diets in lepidopteran species. I want to highlight the importance of taking the natural diet range of insects into account when designing experiments to study host-plant as well as host-plant-pathogen interactions. Additionally, possible long-term effect should be taken into account when applying prophylactic treatments to increase rearing success of laboratory populations.

Four feeding experiments where designed in order to study different aspects of medicative diets and their influence on development, behaviour and insect immunity. All experiments were conducted with larvae of the arctiid polyphagous moth *A. plantaginis*, the two host plants plantain and dandelion, and the opportunistic pathogen *S. marcescens*. The first experiment was established to examine the effect of the two host plants on survival and fitness of the larvae and how they affect their resistance against an infection (chapter I). Further, larvae were individually given the choice to feed on the two host plants in response to an infection, to explore their ability to therapeutically self-medicate (chapter II), and when under a high infection risk, to assess the prophylactic medication ability (chapter III). A fourth feeding experiment was established to examine the long-term effect of a prophylactic medication with an antibiotic on lab-reared larvae (chapter IV). The following hypotheses were addressed in the chapters:

- Polyphagous larvae gain best protection against an infection when switching their diet between two host plants (I)
- Host plants have divergent fitness related effect on polyphagous larvae, which can be seen in their immune response (I)
- Polyphagous larvae therapeutically self-medicate by mixing their diet (II)
- Polyphagous larvae can recognise a high infection risk and in response alter their feeding behaviour to prophylactically medicate (III)
- Larvae avoid feeding on bacterial contaminated diet (III)
- Prophylactic medication can have long-term negative effects for laboratory reared insect populations (IV)

2 MATERIAL AND METHODS

2.1 Study species

2.1.1 Insects, microorganisms, antibiotics

The wood tiger moth, *Arctia plantaginis*, is a day active moth belonging to the *Arctiinae* subfamily, family *Erebidae* (Conner 2008). They are herbivorous, polyphagous insects. The larvae can feed on a variety of plant species, mostly herbaceous and arborescent plants (Ojala *et al.* 2005, Lindstedt 2008). They overwinter as larvae and usually only have one generation per year. When reared under laboratory conditions *A. plantaginis* can run through around three generations per year, and are mostly studied for their warning colouration, but also for immunocompetence questions and host-parasite interactions (Ojala *et al.* 2005, 2007, Friman *et al.* 2009, Lindstedt *et al.* 2009, Zhang *et al.* 2012). The larvae for all experiments were obtained from a stock population at the University of Jyvaskyla, originally initiated from wild caught adults from Estonia. They were all reared under same conditions with a temperature of around 25°C, Light-Dark cycle 18/6 and 80% relative humidity and kept under this conditions for all experiments.

Serratia marcescens is a gram negative enterobacterium with global distribution (Mikonranta 2015). It is an opportunistic pathogen which can be found in different environments, like freshwaters, marine, plants and soil (Grimont and Grimont 2006, Mahlen 2011). *S. marcescens* is known to infect plants, corals, fish, bird, mammals and can be pathogenic in over 70 insect species, which includes various Lepidopteran species (Flyg *et al.* 1980, Grimont and Grimont 2006, Mahlen 2011). The bacterial strain *Serratia marcescens ssp. marcescens db11*, originally isolated from Drosophila melanogaster (Flyg *et al.* 1980), was used for this thesis. Prof. Heinrich Schulenburg, Lauri Mikonranta and Ilkka Kronholm kindly provided the bacterium. The bacterium was cultured in liquid LB-medium (10g tryptone, 5g yeast extract, 10g NaCl in 1L dH^{2}0). Due to its ability to infect plants, it can be assumed that *A. plantaginis* larvae can encounter *S. marcsecens* under natural environmental conditions.

Fumagillin-B is a biomolecule isolated from the fungus *Aspergillus fumigatus*, which is commonly used as antimicrobial agent (Fallon *et al.* 2011, Huang *et al.* 2013, Van Den Heever *et al.* 2014). It is applied in bee-management as well as a substitute for artificial diets for laboratory reared insect species (Frontier Agricultural Science). Fumagillin blocks the enzyme methionine aminopeptidase-2 (MetAP2), which makes it especially effective against microsporidian infections were MetAP2 is an essential enzyme (Upadhya *et al.* 2006, Fallon *et al.* 2011).

2.1.2 Plants

Plantago major (greater plantain) is a herbaceous perennial or annual plant member of the *Plantagiaceae* family, with a worldwide distribution (Chiang *et al.* 2002, Velasco-Lezama *et al.* 2006). Plantain is reported to be used in traditional medicine for wound healing, but is also known for its anti-inflammatory, antimicrobial and antitumor effects (Gomez-Flores *et al.* 2000, Samuelsen 2000, Zhang *et al.* 2012). Leaves, stems and roots contain biologically active compounds like flavonoids, phenols, triterpenes, glycosides and benzoics (Duke 1990, Gomez-Flores *et al.* 2000, Samuelsen 2000, Chiang *et al.* 2002).

Dandelion (*Taraxacum officinale*) is a non-toxic herbaceous perennial plant belonging to the Asteraceae family. With growing habitats on field sides as well as ruderal areas, the plant is distributed all over Europe, Asia and America. Dandelion contains high contents of minerals, fiber, vitamins and fatty acids (Escudero *et al.* 2003). Other than for human nutrition as teas or vegetable, dandelion has long been used in traditional medicine for diuretic and antirheumatic properties but also due to its anti-inflammatory properties and free radical scavenger activity (Williams *et al.* 1996, Hu and Kitts 2003, Schuetz *et al.* 2006, Choi *et al.* 2010).

Dandelion and plantain are both herbaceous plant species, which *A. plantaginis* larvae are known to feed on. Furthermore both plants can grow and occur in same habitats. Knowing that *A. plantaginis* larvae are very mobile and seen to feed on both leaves when given a choice under laboratory condition, we assume that both plants can be natural host diets for them. Plants were collected at different locations around the University of Jyvaskyla, Finland, on daily base.

2.2 Experimental design

2.2.1 Diet switch in groups (I)

To test if the polyphagous *A. plantaginis* larvae need a combination of different diets to gain best development and fitness, they were raised on either dandelion or plantain leaves straight after hatching from egg. They were orally infected with *S. marcescens* for 24h at the age of 4 weeks and divided in 4 diet groups. Two groups were then continuously fed with the same plant and two switched to feed on the other host plant after the oral infection. Larvae were monitored for survival, development and pupa weight. A control group was reared on only plantain or only dandelion during their whole development to examine the effect of host plant.

2.2.2 Individual diet choice (II)

The ability of larvae to actively switch their diet after encountering an infection, was assessed with larvae of *A. plantaginis*. 300 larvae were fed with either dandelion or plantain for 5 weeks. After oral infection with *S. marcescens* they were individually placed in petri dishes, given the choice to feed on plantain or dandelion. Same set-up was performed with non-infected larvae. The individual diet choice was monitored to compare the individual diet choice of infected and non-infected larvae, depending on their initial host plant.

2.2.3 Specificity of diet choice (III)

To assess if *A. plantaginis* larvae can also alter their feeding choice when exposed to a high infection risk, but not yet being infected, 300 5-week-old larvae were placed individually to petri dishes and given the choice to feed on dandelion or plantain. Larvae were initially fed on plantain, because based on results from chapter I feeding on plantain during first instars provided a good protection against infection A high infection risk was introduced by placing with *S. marcescens* killed corpses of *A. plantaginis* larvae to the petri dishes. The diet choice was monitored and compared to individuals that had no corpses in their environment and individuals that were exposed to corpses killed by other than bacterial infection.

2.2.4 Prophylactic long-term treatment with antibiotics (IV)

To study if a long-term prophylactic antibiotic treatment affects lab-reared Lepidoptera species, larvae of *A. plantaginis* with same age were divided into two treatment groups with n=400 individuals each. They were individually reared and offered new food every other day for 4 weeks, introducing them to the prophylactic antibiotic treatment. Larvae were afterwards overwintered for 5 months (4°C, darkness, no food) and then reared until adult eclosure. Survival was monitored during the whole time, weight and developmental time documented and haemolymph samples taken before and after antibiotic treatment for immune assays. Eclosed adults were mated and the egg-laying and larvae hatching documented to compare the mating success between the groups.

2.3 Infection

Larvae were infected or antibiotica treated by oral introduction. Infection with S. *marcescens* was performed by feeding the larvae with bacteria contaminated leaves (I, II, III). The leaves were dipped into overnight cultures of S. marcescens and placed to the rearing containers. After 24 hours the contaminated leaves were removed and the larvae transferred to clean containers (I) or individual petri dishes (II, III). Exposure to fumagillin was also orally introduced, by individually feeding the larvae with leaves dipped into a 1% fumagillin solution (diluted in water) (IV). Control leaves were dipped into water to ensure similar leaf conditions. Larvae were treated for 4 weeks, with fresh contaminated leaves every second day.

2.4 Insect life-history trait measurements

2.4.1 Mortality, developmental time and reproductive success

Mortality was monitored starting 24h after the oral infection was introduced (I, II, III). In chapter I the mortality was checked once a day until adulthood. Individuals of study II were checked for survival daily for 8 weeks until most larvae pupated. Mortality of larvae testing for ability of bacterial avoidance was checked daily for 5 weeks (III). In chapter IV mortality of larvae was checked starting 24h after first exposure to the antibiotic; daily before overwintering and every 2 weeks during overwintering. Dead larvae were removed in all studies.

Date of egg laying, larval hatching, pupation and adult eclosure were recorded to calculate developmental time (I, IV). Reproductive success was assessed by performing single-pair matings with emerged adults within the fumagillin treatment group and control group. Eggs were counted after 3 days and hatched larvae 18 days after egg laying date (IV).

2.4.2 Weight

Weight of larvae was measured before the first antibiotic treatment, 4 weeks after the experiment started (before overwintering) and right after the overwintering period (IV). In addition the weight of pupa was recorded 1 day after pupation (I, IV).

2.4.3 Immune assays

Lysozyme like activity was measured from haemolymph samples collected via pucturing the larvae with a needle. 25 samples were taken from each diet group (dandelion and plantain) before and 24h post oral infection (I). 5µl haemolymph was pipetted in 2mm wells punctured in agar plates (petri dishes (ø 94mm) with 10ml of autoclaved PBS (phosphate buffered saline) mixed with 21 mg *Micrococcus luteus* lyophilisized cells (Sigma), Agar concentration 1.5%). Plates were then incubated overnight at 37°C and photographed. Lytic activity was calculated by measuring the radius of the clear zone around each sample. A standard row was performed by diluting chicken egg white lysozyme (Sigma) to 7 serial dilutions (2 mg/ml, 1 mg/ml, 0.750 mg/ml, 0.500 mg/ml, 0.250 mg/ml, 0.125 mg/ml, 0.62 mg/ml, and 0.31 mg/ml). Furthermore 5µl of haemolymph was mixed in 30µl PBS. Phenoloxidase activity was measured after centrifuging the samples at 4°C for seven minutes at full speed to obtain the supernatant. 20µL of each supernatant was mixed with 100µL 3mM L-Dopa (Sigma, #333786). Changes in absorbance where measured at 30°C and 460nm for 90 minutes with a Victor X4 2030 plate reader (Perkin Elmer, Waltham, MA, US). Same analysis was performed with samples from larvae 4 weeks after prophylactic antibiotic treatment (IV). 4µl haemolymph from 100 individuals from treatment and control group were collected and stored in 100µl PBS.

Concentration of ROS was measured from haemolymph and gut samples taken from 25 individuals from each diet group (dandelion and plantain) before and 24h after oral infection (II). Haemolymph was stored in 60µl PBS and gut samples stored and homogenized in 500µl PBS. The supernatant (gut samples = 400µl, haemolymph samples = 10µl) was used to measure the actual H_2O_2 concentration using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), following the manufactures protocol using an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA). Each sample was run in two technical replicates, and the mean value used as the H_2O_2 concentration for the sample. Additionally also the protein concentration of the gut samples was measured with the Pierce™ BCA Protein Assay Kit (# 23227, Thermo-Fisher Scientific, Waltham, MA, USA) using the same Plate Reader.

3 RESULTS AND DISCUSSION

3.1 Diet switch is of importance for polyphagous larvae (I)

In chapter one it is reported how two different host plants effect development of *A. plantaginis* larvae, and how diet and a mixture of the consumed plants affects the larval ability to survive an infection. Compared to feeding on dandelion, feeding on a monotonous plantain diet decreased the survival of non-infected and infected larvae. In addition, plantain also led to low pupal weight in both infected and non-infected individuals. The developmental time of control larvae is increased when feeding on plantain. Plantain is suggested to have medicational effect, as it contains high amounts of antibacterial compounds (Gomez-Flores *et al.* 2000, Zhang *et al.* 2012). However, the results suggest that a long-term intake of these compounds can have a harmful effect, probably caused by the costs related with the sequestration and detoxification of the allelochemicals (Schoonhoven *et al.* 2005).

Switching the diet from plantain to dandelion had a positive effect on survival of infected larvae, but the opposite diet switch resulted in low survival. The positive effect of the diet switch from plantain to dandelion was also shown as increased pupal weight, compared to a monotonous plantain diet. Other studies showed positive effect of secondary metabolites (as in plantain) when ingested after an infection (Simone-Finstrom and Spivak 2012, M.S. Singer et al. 2014b). Our findings might show that the usage of medicative substances is more complicated than previously thought; emphasizing that diet mixing is of great importance. A switch from a diet rich in secondary metabolites to a diet providing high levels of essential nutrients might be the key to a good resistance against an infection. This specific switch might balance the costs of utilizing the medicative compounds to fight the infections, while switching to plantain after the infection might not provide them the medication effect fast enough. The host plants are not affecting general antibacterial activity in the haemolymph of the larvae, but the production of phenoloxidase is affected.

Individuals feeding on dandelion had a lower phenoloxidase activity in the gut when infected, compared to non-infected larvae.

This study demonstrates that some polyphagous larvae might need a certain diet switch between host plants to gain protection against an infection, though the beneficial effect strongly depends on which order the host plants are ingested. Mixing diets should be taken into consideration when studying host-plant interactions of polyphagous larvae. The results clearly emphasize that switching diets can be important for insects when medicating themselves, which should be assessed in additional food choice experiments.

3.2 Ability of larvae to self-medicate behaviour (II)

Chapter two reports slight evidence for larval ability to self-medicate by mixing their diet; but more importantly highlights that the polyphagous *A. plantaginis* larvae prefer to ingest a mixture of host plants rather then monotonously feeding on one plant. A diet including dandelion, as single host plant or part of a diet mixture, resulted in high survival rates of non-infected larvae. Feeding on a monotonous plantain diet can negatively affect the larva survival. This shows the possible negative effect of a plant high in secondary metabolites for non-infected individuals, fulfilling one criteria for true self-medication (Abbott 2014). From the previous study (chapter I), though, it is known that it might be a diet switch (plantain to dandelion) that actually provides the larvae best protection against an infection.

When given the choice, all larvae clearly selected an intake of both host plants with a higher overall ratio of dandelion. Also larvae initially fed with plantain overall increased the intake of dandelion. This represents the diet switch from plantain to dandelion, which is shown to be the best for infected larvae by chapter I. However, while they overall ingested more dandelion, infected larvae increased the ratio of plantain in their mixed diet compared to control larvae. There was no difference in feeding behaviour of infected and control larvae initially fed with dandelion.

Measurements of reactive oxygen species in the gut and haemolymph of larvae showed that the concentration differs depending on the sampled tissues, being higher in the gut. Furthermore the host plant, which they were feeding on, affected the ROS concentration in the gut. Feeding on plantain increases the amount of ROS in the gut, though the concentration does not differ between infected and non-infected individuals. Insects' immune system produces and releases ROS as a response to an infection. Thus high concentrations of ROS in the gut suggest a up-regulated immune response of the larvae, and accordingly might lead to a deliberate effect on the pathogen (Schmid-Hempel 2005, Kohchi *et al.* 2009, Wu *et al.* 2012). Feeding on plantain might thus help the larvae to fight the infection. After an infection the ROS concentration decreased in the haemolymph of larvae feeding on dandelion, which might lead to a low survival of larvae feeding on dandelion after they encountered an infection.

This result could explain and be in good correlation with the high survival of infected larvae that were switched from plantain to dandelion at the experiments for chapter I.

In conclusion, these results might provide evidence, that *A. plantaginis* larvae can self-medicate by mixing host plants, as some infected larvae showed a different feeding behaviour than non-infected larvae. However, this strongly depends on the host plant that larvae ingest during their early development, or before they encounter an infection, and needs further investigation. While most studies only assess self-medication behaviour with one diet or substance (Chapuisat *et al.* 2007, Singer *et al.* 2009, Manson *et al.* 2010), this study proves that generalist feeders choose to ingest a mixture of host plants to possibly gain best protection against an infection.

3.3 Prophylactic medication (III)

In chapter three it was investigated whether larvae can sense a high infection risk and in response change their feeding behaviour by changing the ratio of the ingested diet mixture of two host plants. Larvae exposed to conspecifics killed by the bacterium *S. marcescens* showed a different feeding behaviour than larvae exposed to conspecifics killed by freezing (low infection risk) and larvae without any conspecifics in their environment. Based on the results of chapter II it was expected that larvae (initially fed on plantain) in a high infection risk would choose to increase the intake of plantain to their mixed diet compared to larvae in the low infection risk. All larvae did choose to ingest a mixture of both host plants, and other than expected, larvae in high infection risk ingested the highest ratio of dandelion. As the larvae were fed on plantain before exposed to the different treatment, the change to feed on dandelion might represents the beneficial diet switch from plantain to dandelion for infected larvae shown in chapter I. In chapter II, studying therapeutic medication, infected larvae initially fed on plantain, showed an increased plantain intake compared to control larvae, while still overall ingesting more dandelion. This shows a different feeding behaviour for larvae depending on whether they are infected or only exposed to a risk of infection. The larvae might not have been exposed to a severe enough infection risk, not triggering a change of feeding behaviour to increased intake of plantain. It was seen, that after 10 days of monitoring larvae in the high infection risk environment started to increase the intake of plantain. The infection risk might have been become bigger with time, and when monitoring the larvae for longer time, we might have seen the same feeding behaviour than in chapter II. Before the infection risk gets sever, the costs for ingesting plantain, and thus to sequester and detoxify the allelochemicals might be too high while the infection risk is too low. However, the results are in perfect accordance with the results form chapter I, showing the beneficial effect of a diet switch from plantain to dandelion.

This study provides evidence for the larva ability to sense a high infection risk, though it remains discussable whether the monitored change of feeding behaviour is a case of prophylactic medication. While the increased intake of dandelion after feeding on plantain, might show a case of prophylactic medication based on the beneficial diet switch shown in chapter I, it could also represent a case of prophylactic consumption. In the latter case, there is no detrimental effect of the ingested substance for the organisms itself, like it is induced by a plantain diet for non-infected larvae. Here, the larvae in high infection risk environment did not ingest more plantain compared to the other treatment groups and the results might thus indicate prophylactic consumption by *A. plantaginis* larvae.

Larvae did not avoid feeding on with bacteria covered leaves, when given the choice. While they seem to be able to recognise corpses killed by the pathogen, they maybe can not sense leaves contaminated with the pathogen. This suggests, in accordance with a study by Capinera et al., that larvae do not react to the single cue of the bacteria but rather to a combination of the bacteria and fluids released by the cadaver (Capinera *et al.* 1976). However, the LB medium could also have affected the larvae, by its smell or a possible contamination or settlement of bacteria during the monitoring period.

3.4 Long-term effect of prophylactic treatment (IV)

Chapter four shows the long-term effect of a prophylactic antibiotic treatment on lab-reared *A. plantaginis*. A prophylactic treatment with fumagillin did not affect the survival of larvae during overwintering phase. In addition, the treatment had no negative effect on larval development and weight. In contradiction to the results found by Rutledge, who found a negative effect on fumagillin on development on mosquitos (Rutledge 1970), our results did not indicate a similar effect. The prophylactic treatment of mass reared lepidopteran with fumagillin might thus be a good decision, as it does not affect developmental time and overwintering ability. Nevertheless, individuals treated with fumagillin showed a lower reproductive success, as they laid fewer eggs than control individuals. This is in good correlation with negative effects of fumagillin on brood production of *Bombyx occidentalis* and *Apis mellifera* (Webster 1994, Whittington and Winston 2003). Guaranteeing and maintaining a good reproductive success is of key importance for lab-reared insects.

In conclusion, prophylactic medication of lab-reared insects should be carefully considered. While there is no effect on survival and development, it has a decreased reproductive success, and thus might have severe long-term effect for lab-reared populations.

4 CONCLUSION

I aimed to study the different effects of host plants on polyphagous larva of *A. plantaginis*, and how diet affects their fitness. Furthermore I wanted to study their ability to use different plant to medicate themselves in response to an infection, with special emphasizes on the importance of a mixed diet as medicine.

The first question was whether host plants have a divergent fitness related effect on polyphagous larvae, reflected in their immune responses. The results of chapter I and II show that plantain and dandelion indeed differently affect larvae, with plantain overall seeming to be harmful when ingested monotonously and over long period. Surprisingly, immunocompetence was not as strongly affected by the specific host plants as expected and seems to not have a strong influence on the different effects of the plants on the larval ability to survive an infection. Next interesting question was whether the polyphagous larvae gain best protection against an infection when switching their diet between two host plants. This kind of diet switch represents the natural feeding behaviour of many generalist feeders, but is to our knowledge not well studied in relation to insects' ability to resist an infection. Results of chapter I show, that a diet switch between the two plants resulted in best survival of infected larvae. Interestingly a diet switch can have opposing effects, depending on which plant is ingested before and after the infection. It might be of key importance for some polyphagous insects to ingest a mixture of host plants when infected.

After understanding which diet is best for the polyphagous *A. plantaginis* larvae when infected, I asked whether they also would actively ingest the certain beneficial diet mixture after getting infected. Larvae overall prefer to ingest more dandelion when given the choice, but do not switch to feeding monotonously on one plant. This highlights again the relevance of the natural diet breadth of polyphagous larvae when studying them. Infected larvae initially fed on plantain ingested more plantain than control individuals, which could indicate a case of medication behaviour. However, the full evidence for a case of therapeutic self-medication, the active contact of a diet high in biologically active compounds in response to an infection, could not be provided.

Self-medication occurs therapeutically after an infection, but is also done prophylactically in order to avoid getting sick. After knowing the different effect of host plants, and gaining evidence for larval ability to actively switch their feeding behaviour depending on their infection status, I asked whether they also alter their feeding behaviour to prophylactically medicate and can sense the pathogen in their environment. Larvae did not avoid feeding on bacteria contaminated leaves, though they seem to respond to cadavers of conspecifics killed by the pathogen. Feeding behavior of larvae exposed to a high infection risk environment (corpse killed by the pathogen) showed a different feeding behavior compared to other treatment groups, which might prove their ability to respond to a high risk of getting infected in their environment. They ingested overall the highest ratio of dandelion, but all larvae again did not choose to feed solely on one plant (chapter III). Further studies with other host plants and a higher infection risk exposure need to be conducted and will help to understand and extend the knowledge about insects ability to use prophylaxis within insect-plant interactions. I can demonstrate the evidence for prophylactic behavior in larvae and beneficial effect of the following diet change.

The last question I asked was whether a prophylactic treatment can have long-term negative effects for lab-reared insects and the results revealed, disadvantages of prophylaxis (chapter IV). A medical prophylactic treatment with an antibiotic did not affect the survival of larvae, nor their overwintering ability and development. However, the prophylactic treatment had a negative effect on the larvae long-term fitness, reducing their reproductive success. Prophylaxis in lab-reared insects should thus be well considered.

All together, the results of this thesis reveal how important it might be to take into account the natural diet range of polyphagous insects when studying host-plant interactions. It emphasizes the importance of a balanced diet for polyphagous *A. plantaginis* and the complexity of diet switching when medicating in response to an infection. Further studies with other host plants, more polyphagous insect species and a higher infection risk exposure need to be conducted and will help to understand and extend the knowledge about insects ability to self-medicate and the importance of host plants on their development and resistance against infections.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Ruoan ennaltaehkäisevä vaikutus polyfaagin perhosen *Arctia plantaginis* (Arctiidae) elinkykyyn ja immuniteettiin

Hyönteisten kelpoisuus riippuu monista eri tekijöistä, joista tärkeimpiä ovat ravinto ja taudinaiheuttajat. Ravinto, sen laatu ja määrä on yksi tärkeimmistä ekologisista tekijöistä, jotka vaikuttavat yksilöiden kasvuun, kehitykseen, lisääntymiseen, elinikään ja immuunivasteeseen. Lisäksi ravinto voi vaikuttaa hyönteisten kelpoisuuteen epäsuorasti lisäämällä infektio- tai loisriskiä, sekä riskiä joutua saalistuksen kohteeksi. Kasvinsyöjähyönteiset altistuvat myös erilaisille kasvien tuottamille biokemiallisille yhdisteille (allelokemikaalit). Hyönteisille onkin kehittynyt monia puolustusmekanismeja infektioita ja ravinnon sisältämiä kemikaaleja vastaan. Näistä tärkeimpiä ovat morfologiset esteet kuten kuoripanssarit tai solujen puoliläpäisevät kalvorakenteet. Hyönteisillä on myös varsin kehittynyt immuunijärjestelmä, joka on yksi tärkeimmistä infektionjälkeisistä puolustusmekanismeista. Tämän lisäksi myös yksilön käyttäytymiseen perustuvat puolustuskeinot ovat tärkeitä sekä ennen infektiota että sen jälkeen. Näihin kuuluvat infektoituneen ruoan tai lajikumppanien välttäminen, puhtaudesta huolehtiminen ja ravinnonhankinnan ajoituksen tai munintapaikan sijainnin muuttaminen. Eräs viimeaikoina suurta huomiota saanut keino on itselääkintä, jolla tarkoitetaan hyönteisten kykyä valita tai muuttaa ravintoaan vasteena infektioon. Tämä voi tapahtua joko ennen infektiota (ennaltaehkäisevä itselääkintä) tai infektion jälkeen (hoidollinen itselääkintä).

Hyönteisten kykyä itselääkintään on testattu useissa tutkimuksissa. Nämä ovat kuitenkin keskittyneet joko yhden ravintoaineen vaikutuksiin tai vain yhteen ravintokohteeseen perustuvaan ruokavalioon siirtymiseen. Hyönteisten luontaisen ruokavalion laajuus on sen sijaan jäänyt vähemmälle huomiolle. Vaikka hyönteisten ravitsemustarpeet ovatkin usein samankaltaisia, ne voivat vaihdella tiettyyn ravintokohteeseen erikoistuneiden lajien (spesialistit) ja useita ravintokohteita hyödyntävien lajien (generalistit) välillä. Jälkimmäisiä kutsutaan myös polyfaageiksi. Nämä hyönteiset pystyvät hyödyntämään ravintokasveja useista eri kasviheimoista, jotka vaihtelevat sekä ravinnepitoisuuksien että sekundaaristen aineenvaihduntatuotteiden osalta. Tämän johdosta ravintokohteiden vaihtelu saattaisi olla paras strategia minimoimaan sekundaaristen aineenvaihduntatuotteiden haitalliset vaikutukset sekä varmistamaan riittävä ravinteiden saanti. Ravintokasvien vaihtelun on osoitettu lisäävän kasvinsyöjähyönteisten kelpoisuutta ja lisäksi tiedetään, että jotkin hyönteislajit vaihtavat isäntäkasvejaan luonnossa säännöllisesti. Ravintokohteiden vaihtaminen vaikuttaisikin olevan tärkeä tekijä polyfaagien hyönteisten itselääkintää tutkittaessa.

Väitöskirjassani selvitin kahden eri isäntäkasvin vaikutusta polyfaagiin täpläsiilikäs perhoseen, *Arctia plantaginis*, tutkimalla ravintokasvin vaihtamisen merkitystä sekä infektioilta suojautumiseen että yksilöiden kelpoisuuteen. Lisäksi tavoitteenani oli saada uutta tietoa siitä, miten perhoset voivat lääkitä itseään ravintokohteita vaihtamalla. Haluan myös korostaa hyönteisten luonnollisen ruokavalion laajuuden huomioimista kasvinsyöjien, kasvien ja patogeenien välisiä vuorovaikutuksia tutkittaessa. Lisäksi myös mahdollisten pitkäaikaisvaikutusten huomioiminen on tärkeää, jos ennaltaehkäisevää hoitoa halutaan soveltaa laboratoriopopulaatioiden kasvatuksessa.

Täpläsiilikäs toukalla on useita isäntäkasveja, joista voikukka (Taraxacum sp.) ja ratamo (Plantago major) vaikuttavat eri tavoin yksilöiden kelpoisuuteen. Voikukassa on korkea ravintopitoisuus, kun taas ratamo on tunnettu sen korkeista sekundaaristen aineenvaihduntatuotteiden pitoisuuksista. Pitkällä aikavälillä pelkkää ratamoa sisältävällä ruokavaliolla vaikutti olevan haitallisia vaikutuksia täpläsiilikkään kasvuun ja elinkykyyn. Sen sijaan voikukan lisääminen ruokavalioon lisäsi yksilöiden selviytymistä infektion jälkeen ja vaikutti positiivisesti yksilöiden kehitykseen. Parhaiten selvisivät toukat, jotka siis saivat sekä ratamoa että voikukkaa, tässä järjestyksessä. Ravintokohteen vaihtaminen voikukasta ratamoon sen sijaan johti yllättäen infektoituneiden toukkien korkeaan kuolleisuuteen. Tämä viittaa siihen, että pelkästään ravintokasvien vaihtelun sijaan tärkeämpää polyfaageille hyönteisille olisi siirtyä tietystä ravintokasvista toiseen oikeassa elinkierron vaiheessa. Vaikka eri kasveista koostuvat ruokavaliot vaikuttivat toukkien kehitykseen, ne eivät vaikuttaneet yksilöiden immunopuolustukseen.

Selvitettyäni, miten ravintokasvien vaihtaminen vaikutti täpläsiilikkään toukan selviytymiseen infektion jälkeen, seuraava kysymys oli, osaisivatko infektoituneet toukat vaihtaa isäntäkasviaan ravintokasville, joka antaa niille parhaan suojan. Toukat saattaisivat suojautua infektioilta parhaiten lisäämällä ravintoonsa ratamoa sen sisältämien sekundaaristen aineenvaihduntatuotteiden johdosta. Tämän johdosta ratamolla saattaisi olla lääkitseviä vaikutuksia ja sen lisääminen ruokavalioon voisi olla hyödyllistä toukkien itselääkinnässä. Kun toukkien annettiin valita kahden ravintokasvin välillä, yksilöt söivät enemmän voikukkaa. Toukat eivät kuitenkaan siirtyneet pelkästään voikukkaa sisältävään ruokavalioon, vaan suosivat molempien ravintokasvien yhdistelmää. Infektoituneet toukat, joita oli aiemmin ruokittu ratamolla, suosivat ratamoa enemmän kuin kontrolliryhmään kuuluvat yksilöt. Tämä viittaa itselääkintään, jolla tarkoitetaan aktiivista ruokavalion muuttamista vasteena infektioon.

Tulokseni siitä, että polyfaagit toukat voivat lääkitä itseään yhdistelemällä molempia ravintokasveja ruokavalioonsa, herätti kysymyksen, voisivatko toukat muuttaa ruokavaliotaan myös ennaltaehkäisevästi. Tämän avulla yksilöt voisivat välttää tartunnan tunnistettuaan korkean infektioriskin. Yksilöt voisivat myös mahdollisesti tunnistaa pilaantuneen ravinnon ja välttää sitä. Tulokseni osoittivat, että toukat eivät osanneet välttää bakteereita sisältäviä lehtiä. Sen sijaan toukat muuttivat ruokavaliotaan, kun lähistöllä oli patogeenien tappamia yksilöitä. Nämä yksilöt suosivat voikukkaa enemmän kuin kontrolliryhmä, jota ei oltu altistettu korkealle infektioriskille. Samoin kuin aiemmissa kokeissa, kaikki toukat kuitenkin suosivat ravinnossaan molempien kasvien yhdistelmää, koeryhmästä riippumatta. Vaikka korkealle infektioriskille altistetut toukat eivät muuttaneet ruokavaliotaan samalla tavalla kuin infektoituneet toukat (syömällä enemmän ratamoa), näiden yksilöiden ruokavalio erosi toukista, joilla ei ollut korkeaa infektioriskiä. Tämä viittaa siihen, että toukat pystyvät tunnistamaan patogeenin ympäristössään.

Tämän lisäksi tutkin ennaltaehkäisevän hoidon mahdollisia pitkäaikaisvaikutuksia laboratoriossa kasvatettuihin *täpläsiilikkään* toukkiin. Ennaltaehkäisevien antibioottien lisääminen laboratoriossa kasvatettavien hyönteisten ravintoon on yleistä, mutta antibioottien mahdollisia haittavaikutuksia ei ole kuitenkaan tutkittu kattavasti. Nämä haittavaikutukset eivät välttämättä ilmene välittömästi, vaan saattavat vaikuttaa vasta pitkän aikavälin jälkeen. Tulokseni osoittivat, että ennaltaehkäisevällä antibioottilääkityksellä ei ollut vaikutusta toukkien selviytymiseen, talvehtimiskykyyn tai kehitykseen. Sen sijaan sillä oli negatiivinen vaikutus pitkäaikaiseen kelpoisuuteen, sillä se heikensi yksilöiden lisääntymismenestystä. Tämän johdosta ennaltaehkäisevän lääkityksen käyttöä laboratorioissa kasvatettuihin hyönteispopulaatioihin pitäisikin harkita tarkkaan.

Väitöskirjani tulokset osoittavat, että isäntäkasvien ja kasvinsyöjien vuorovaikutuksia tutkittaessa on tärkeää ottaa huomioon polyfaagien hyönteisten luonnollinen ravintovalikoima. Tulokseni myös korostavat monipuolisen ruokavalion tärkeyttä polyfaageille hyönteisille sekä osoittavat, että ravintokohteiden muuttaminen vasteena infektioon voi olla luultua monimutkaisempaa. Jotta hyönteisten kykyä itselääkintään, ja isäntäkasvien merkitystä yksilöiden kehitykseen ja resistanssiin infektioita vastaan ymmärrettäisiin paremmin, olisi tulevissa tutkimuksissa tärkeää testata tätä eri isäntäkasveilla ja hyönteislajeilla, sekä altistamalla yksilöt korkeammalle infektioriskille.

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LONG-TERM PROPHYLACTIC ANTIBIOTIC TREATMENT: EFFECTS ON SURVIVAL, IMMUNOCOMPETENCE AND REPRODUCTION SUCCESS OF *PARASEMIA PLANTAGINIS* (ARCTIINAE)

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Research article

Long-Term Prophylactic Antibiotic Treatment: Effects on Survival, Immunocompetence and Reproduction Success of *Parasemia plantaginis* (Lepidoptera: Erebidae)

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Abstract

Hundreds of insect species are nowadays reared under laboratory conditions. Rearing of insects always implicates the risk of diseases, among which microbial infections are the most frequent and difficult problems. Although there are effective prophylactic treatments, the side effects of applied antibiotics are not well understood. We examined the effect of prophylactic antibiotic treatment on the overwintering success of wood tiger moth (*Parasemia plantaginis*) larvae, and the postdiapause effect on their life-history traits. Four weeks before hibernation larvae were treated with a widely used antibiotic (fumagillin). We monitored moths' survival and life-history traits during the following 10 mo, and compared them to those of untreated control larvae. Prophylactic antibiotic treatment had no effect on survival but we show effects on some life-history traits by decreasing the developmental time of treated larvae. However, we also revealed relevant negative effects, as antibiotic treated individuals show a decreased number of laid eggs and also furthermore a suppressed immunocompetence. These results implicate, that a prophylactic medication can also lead to negative effects on life-history traits and reproductive success, which should be seriously taken in consideration when applying a prophylactic treatment to laboratory reared insect populations.

Key words: mass-rearing, antibiotic, reproduction, immunocompetence, trade-off

Mass-rearing of insects under laboratory conditions is a widely used strategy in a variety of disciplines, but complications often arise while optimizing the procedure. Insect rearing is important for many different substantial research purposes such as the production of chemical insecticides, agricultural health research, pest management, genetic studies, and enhancement of domestic populations (Leppla 2009, Sørensen et al. 2012). Due to the raising interest on integrated and biological pest management methods, it is of special importance to maintain high quality laboratory populations with minimized workload (Singh 1982, Sørensen et al. 2012). However, it is also known that conditions in laboratory facilities can have negative effects on insects' fitness and reproductive success, as insects' performance can be affected by various behavioral, reproductive, or genetic factors (Singh 1982, Sørensen et al. 2012). The rearing of well-established laboratory model organisms like the confused flower beetle *Tribolium confusum*, the housefly *Musca domestica*, or the tobacco budworm *Heliothis virescens* is well recorded by freely available rearing protocols (Leppla 2009), though the number of scientifically based rearing protocols is humble (Cohen 2001). Establishing new insect species as laboratory populations is highly time consuming (Leppla 2009) due to work needed for the optimisation of rearing techniques, which is necessary to minimize the negative effects of the artificial rearing conditions.

The main limiting factor of artificially reared insect quality is, besides temperature and humidity conditions, a suboptimal nutrition that can consequently lead to microbial contaminations of the populations (Sikorowski and Lawrence 1994), which are known as major threats of low overwintering survival and reproduction success (Rull et al. 2005; Van Der Hoeven et al. 2008; Sørensen et al. 2012). Especially under laboratory conditions infections and diseases can easily establish and spread, affecting sensitive life stages such as overwintering, which is strongly influenced by light- and temperature conditions as well as by food quality (Bale and Hayward 2010, Xu et al. 2011, Spurgeon 2012, Sinclair 2014). Insect mating behavior is dependent on various environmental factors, but also on courtship behavior and fitness, and is furthermore affected by diseases (Leppla 2009). Production of high numbers of high quality insects might be achieved by including a prophylactic microbial control treatment (Singh 1982, Parker et al. 2005, Sørensen et al. 2012). Developing and understanding the importance of artificial diets and optimal rearing conditions is essential to guarantee high quality insect populations as well as the applicability of the results obtained from experiments (Cohen 2000, 2001; Leppla 2009; Sørensen et al. 2012).

The use of prophylactic antibiotic treatments in laboratory-reared insect populations is a commonly used strategy to eliminate microbial infections and increase the quality of mass-reared insects (Sørensen et al. 2012). Laboratory-reared colonies of Lepidoptera (McLean-Cooper et al. 2008, Van Der Hoeven et al. 2008), Diptera (Dimou et al. 2010), and Coleoptera (Lehman et al. 2009) are fed on artificial diets complemented with antibiotics to eliminate epidemic infections such as contamination with obligate intercellular parasites, for example microsporidia (Higes et al. 2007). They can affect insect life-history traits negatively by a restricted larval development, decreased pupal weight, lower fecundity, and immune suppression, as detected for instance in *Choristoneura fumiferana* (Thomson 1958) and *Apis mellifera* (Antúnez et al. 2009). These effects could render insects more susceptible to secondary and/or opportunistic infections. Moreover, it is known that the adaptation to chemicals is associated with fitness costs, which may also include suppression of the immune response, as it is a costly mechanism (Coustau et al. 2000, Schmid-Hempel 2005). Insect immune system is a well-evolved defense against infections, consisting of complex multi-level interactions between specific detoxification enzymes and genes (Gillespie et al. 1997, Vilmos and Kurucz 1998). Up-regulating and maintaining an immune response is highly costly and thus trades off with other cost-associated factors, such as environmental stressors and diseases (Moret and Schmid-Hempel 2000, Schmid-Hempel 2003).

A commonly used antibiotic reagent for prophylactic therapy against microsporidia infection is fumagillin, isolated from the fungus *Aspergillus fumigatus* (Huang et al. 2013, Van Den Heever et al. 2014). For example the leading company of insect supplies (Frontier Agricultural Science) is offering ready mixed insect diets supplemented with antibiotics. However, there is little known about the long-term effects of the applied agents. Possible negative effects on life-history traits are suspected but still not well documented. Prolonged larval, pupal, and adult developmental times, as well as decreased reproductive success, are possible long-term consequences of a prophylactic treatment of laboratory insect populations (Wilkinson 1998). Wild insect populations are also facing increasing amounts of different xenobiotics. With the increased usage of antibiotics in human medical treatment, as well as in food animal production, insects might encounter their residuals, which stay active in environmental compounds (Daghrir and Drogui 2013).

The main objective of this study was to test the effect of a prophylactic antibiotic treatment on life-history traits and overwintering success of Lepidopteran larvae not showing obvious symptoms of an infection or disease, as well as the general long-term consequences of the medicine. Polyphagous larvae of the wood tiger moth, *Parasemia plantaginis* where used for this experiment. We treated the larvae by feeding them with the antibiotic fumagillin for a time span of 4 wk until they began to hibernate. We then monitored larval mortality before hibernation as well as 5 mo during hibernation. Furthermore, we examined the effect of the antibiotic on the immune response of the larvae before hibernation, by analyzing the activity of phenoloxidase. The possible long-time consequences of the medicine where assessed in several ways: 1) we measured the weight of the larvae before hibernation and after hibernation, as well as the pupal weight; 2) we monitored the developmental time from egg to pupa, and from egg to adult; 3) we examined the effect on egg laying success and on the number of laid eggs after mating the eclosed adults. Our findings offer a better understanding of the side effects that the treatment with prophylactic medicine has on laboratory-reared Lepidoptera larvae.

Material and Methods

Animals

The wood tiger moth, *P. plantaginis*, is a day active moth belonging to the Arctiinae subfamily (Conner 2008). They are herbivorous generalistic insects, which are able to consume and digest a variety of plant species. *P. plantaginis* is widely distributed over the northern hemisphere and is mostly studied for its warning coloration (Lindstedt et al. 2009, Hegna et al. 2015), but also for immunocompetence questions and host–parasite interactions (Ojala et al. 2005, Friman et al. 2009, Zhang et al. 2012, Nokelainen et al. 2013). Under natural conditions they overwinter as larvae and have one generation per year, whereas under laboratory conditions it is possible to have up to three generations per year (Ojala et al. 2007).

All *P. plantaginis* larvae used in this experiment were obtained from a laboratory stock population from the University of Jyväskylä, Finland (re) established in 2012. The laboratory stock is reared under greenhouse conditions of 25°C, a photoperiod of 18:6 (L:D) h, 80% RH, maintained in plastic boxes in groups of around 30 individuals, and fed with dandelion (reared following the methods from Lindstedt et.al [2009]). All larvae used for this experiment had the same hatching date, 16 September 2013. The individuals for the experiment were maintained continuing the above-mentioned rearing conditions.

Fumagillin

For conducting the experiment we obtained the product fumagilin-B (hereafter always referred as fumagillin), a soluble powder from Medivet (Medivet Pharmaceuticals Ldt., High River, Alberta, Canada), which is equivalent to 21 mg fumagillin base per gram. Fumagillin is a commonly used antimicrobial agent in bee-management and human medicine (Huang et al. 2013, Van Den Heever et al. 2014). It is a complex biomolecule isolated from the fungus *A. fumigatus*. Due to its ability to inhibit and block the enzyme methionine aminopeptidase-2 (MetAP2) it is widely used in human medicine to treat microsporidian infections (Fallon et al. 2011). MetAP2 is an essential enzyme in microsporidia and thus its inhibition by fumagillin kills microsporidian cells (Upadhya et al. 2006). The most relevant field of fumagillin application is in beekeeping management, as this substance is proved to be highly effective against nosema diseases in honeybees, *A. mellifera*. Both *Nosema apis* infection, as well as the microsporidian pathogen, *Nosema ceranae*, can be treated with a periodic fumagillin treatment (Webster 1994, Huang et al. 2013). Hives are treated in autumn and spring to ensure microsporidia-free colonies by prophylactically applying a recommended concentration of 25 mg/l of fumagilin in sugar syrup (Huang et al. 2013).

Treatments

Larvae for the experiment were taken from four families of the Finnish laboratory stock population in Jyväskylä with same hatching date (16 September 2013). Twenty-five-day-old larvae were divided into two treatment groups and placed individually in petri dishes (Sarstedt AG & Co, Nuernbrecht, Germany), resulting in 400 antibiotic treated and 400 control treated individuals. Because of uneven development within the families, the number of larvae per family could not be perfectly balanced, resulting in a sample size of 150, 330, 190, and 130 in families 1, 2, 3, and 4, respectively. In order to induce diapause, the temperature and light conditions of the growth chamber where stepwise decreased every week (first week 20°C and a photoperiod of 16:8 (L:D) h, second week 16°C and a photoperiod of 12:12 (L:D) h, third week 12°C and a photoperiod of 8:16 (L:D) h, fourth week 8°C and a photoperiod of 4:20 (L:D) h). For the antibiotic treatment we used 1% fumagilin solution in water. Larvae were orally treated with the antibiotic by dipping the food plant into the fumagillin solution. The treatment lasted for 4 wk. The procedure was repeated every second day to ensure a continuous exposure with the antibiotic. Remaining diet from the last inoculation was removed. Control larvae were treated with the same method, by using water as dipping solution for the food plant, to ensure similar leaf conditions. After 4 wk all larvae were transferred to individual overwintering containers, filled with moss and stored in a climate chamber with 4°C in complete darkness for hibernation. Larvae were kept for 5 mo under hibernation conditions and then placed in a warmer climate chamber (7°C) with increasing temperature and light conditions to slowly wake them up (7°C and a photoperiod of 8:16 (L:D) h cycle with low light intensity (2 out of 5, light intensity level), 4 d after waking up 15°C and a photoperiod of 16:8 (L:D) h cycle, 6 d after waking up 20°C and a photoperiod of 16:8 (L:D) h cycle with high light intensity (4 out of 5), 10 d after waking up greenhouse conditions of around 25°C). Individual rearing was then changed to group rearing, wherefore the larvae were placed in new bigger rearing containers according to their weight, family, and treatment. This grouping allowed further assignment of the larvae to its previous treatment group (fumagillin or control) as well as family. Overall 27 containers with control larvae and 28 containers with fumagillin treated larvae were kept for further observation. Gender was determined in pupa stage.

Survival

The survival was checked during the whole period of individual rearing. We monitored daily survival of individually reared larvae from both fumagillin and control treatments during the 4 wk prior to overwintering. During overwintering, larval mortality was checked every 2 wk and dead animals were removed. The survival monitoring ended with the start of group rearing after the 10 d waking-up period following the overwintering phase.

Immunity

Four weeks after the first fumagillin treatment (see Supp Fig. 1 [online only]) hemolymph from 100 individuals from both treatment groups was sampled. Hemolymph was collected by puncturing the larvae with a sterile needle. Four microliters of hemolymph were immediately mixed with 100 µl chilled phosphate buffered saline buffer and stored at −80°C until further use. For estimating the Phenoloxidase activity samples were thawed on ice and then centrifuged at 4°C for 7 min at full speed to obtain the supernatant. The assay was performed in a 96-well plate. Twenty-four microliters of each supernatant were mixed with 200 µl 3 mM L-Dopa (Sigma Aldrich, Helsinki, Finland). To analyze the phenoloxidase activity, changes in absorbance where measured at 30°C and 490 nm for 90 min with a Victory X4 2030 plate reader (Perkin Elmer, Waltham, MA, USA).

Development

Developmental time. To control the effect of fumagillin on developmental time of *P. plantaginis*, every developmental stage was monitored and dates documented. The egg-laying date was used as the starting date for development. During the whole experimental process larval hatching date, pupation date, as well as adult eclosion date were monitored and recorded. These data were further used to calculate the developmental time from egg to pupa and from egg to adult and thus the effect of the fumagillin treatment on the development of *P. plantaginis* larvae could be examined.

Weight. Larval weight was measured on three different time points before pupation; pupa weight was measured too (see Supp Fig. 1 [online only]). The first weight measurement was taken on 25-d-old larvae before the treatment started; the second one was taken 4 wk later, and the third one right after the overwintering period. Pupa weight was measured 1 d after pupation.

Egg laying and hatching. To examine the egg laying and hatching-success single-pair matings were performed with the emerged adults, within families and within the treatment groups. In total, 24 matings were conducted within the antibiotic treatment group and 15 matings in the control group. Pairs were placed together in plastic boxes (12 by 10 by 10 cm) under above-mentioned rearing conditions (25°C, a photoperiod of 18:6 (L:D) h, 80% RH). After 3 d all eggs were counted. Additionally hatched larvae were counted after 21 d (18 d after egg counting).

Statistical Analyses

All statistical analyses were performed with R 3.1.1 (R Foundation for Statistical Computing, 2014, Vienna, Austria).

All data were checked for normality and homogeneity of variance, and family was used as a random factor. Reported error terms are standard deviations, unless specified otherwise. We analyzed the survival upon treatment separately for the time before and during hibernation with Cox Proportional Hazard-Models. Treatment was added as a fixed factor and family as a random effect. We used analysis of variance (ANOVA) to test for differences in larval weight gain across treatment and developmental time point (day). To account for differences in larval weight before the experiment, we subtracted the weight of first weight measurement from the weights of second and third weight measurement after treatment. Larval development was calculated as timespan between egg laying date and pupation date; overall development was calculated as timespan between egg laying date and adult date. We analyzed each development characteristic with a separate ANOVA, with treatment and gender as fixed factors, and an interaction term of the two factors. Reproductive success was analyzed as the amount of laid eggs per mating couple with a Zero inflated Count Model. The Zero inflated Count Model performed a binomial test on the probability to lay eggs among treatment and a Poisson regression on the amount of laid eggs per treatment. Furthermore a generalized linear mixed model via penalized quasi-likelihood (PQL) was used for examining the proportion of hatched larvae depending on the amount of laid eggs and treatment. Data of immune parameters were analyzed with a Mann–Whitney test, using treatment as factor.

Results

Survival

Treatment effect on larval survival before and during hibernation. A prophylactic treatment with fumagillin did not increase *P. plantaginis* survival during overwintering in comparison to control larvae (Fig. 1: Cox proportional-hazard regression; $\beta = 0.06 \pm 0.14$ (se),

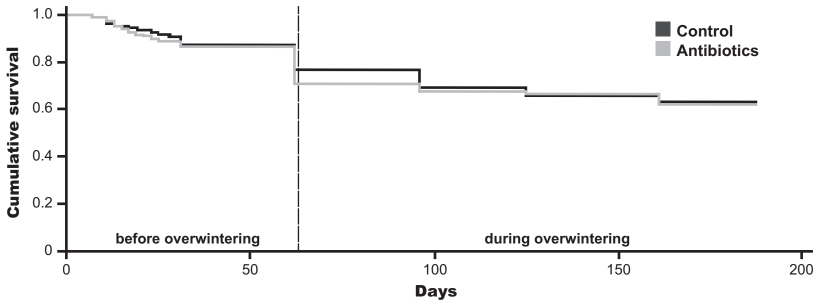

Fig. 1. Effect of fumagillin treatment on larval survival of *P. plantaginis* before hibernation (50 d) and during hibernation compared to survival of control larvae. Dashed line separates survival curve in before overwintering mortality (left) and mortality during overwintering (right).

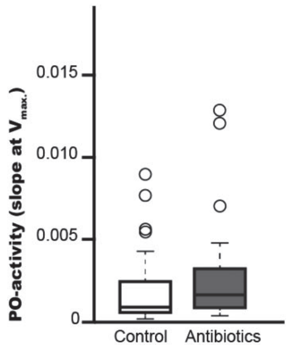

Fig. 2. Phenoloxidase activity in the hemolymph of *P. plantaginis* larvae, fed on fumagillin, 4 wk after treatment exposure, compared to control treated larvae of same age. Phenoloxidase activity (slope at Vmax.) measured from hemolymph samples, comparing the activity of antibiotic treated larvae and control larvae.

$\chi2 = 0.17$, $P = 0.68$). Furthermore, the treatment did not affect the survival before the start of hibernation (Fig. 1: Cox proportional-hazard regression; $\beta = 0.09 \pm 0.2$ (se) $\chi2 = 0.2$, $P = 0.65$)

Immunity

Phenoloxidase activity. Larval phenoloxidase activity in the hemolymph was significantly higher in the fumagillin treatment group than in the control group (Fig. 2: Wilcox-test: W = 2271, $P = 0.007$).

Development

Effect of fumagillin on developmental time of P. plantaginis Developmental time was calculated as the larval development (timespan from egg-laying date until pupation date) and also as the overall developmental time (timespan from egg-laying date until adult eclosion date). Development differs between the genders, with males showing a shorter developmental time (Table 1: mean days to pupation: males = 237.95 ± 2.63; females = 240.46 ± 2.92). Fumagillin treated larvae develop faster than control treated larvae (Table 1: mean days to pupation: control = 239.49 ± 3.0; fumagillin = 238.53 ± 2.95). However, there is no effect of fumagillin on the overall developmental duration from egg to adult (Table 1: mean days to eclosion: control = 248.73 ± 3.59; fumagillin = 248.05 ± 3.54). There is no difference in sex ratio (appearance of male and female pupae) and fumagillin does not affect the survival of male and female pupae differently ($\chi2$ test: $x^2 = 0.003$, df = 1, $P = 0.955$).

Table 1. Effect of fumagillin treatment on the developmental time of *P. plantaginis*

Source of variation	df	MS	*F*	*P*
Developmental time				
egg to pupa				
treatment	1	58.3	7.296	0.007
gender	1	333.7	45.274	1.556e−10
treatment:gender	1	2.6	0.354	0.552
residuals	213	7.4		
egg to adult				
treatment	1	12.35	1.463	0.228
gender	1	55.48	6.573	0.011
treatment:gender	1	4.63	0.548	0.460
residuals	198	8.44		

Results of ANOVA testing for the effect of treatment, gender and their interactions on developmental time measured as time in days from egg to pupa and egg to adult (df = degrees of freedom; MS = Mean Square, *F* = *F*-value; *P* = significance probability).

Effect of fumagillin on larval and pupal weight. Compared to control larvae, there is no significant weight gain or loss of fumagillin treated larvae neither during overwintering (Table 2: standardized larval weight: control = 1.44 ± 8.81 g; fumagillin = 1.31 ± 9.78 g), nor at the two time points (Table 2: standardized larval weight: second weighing = −1.36 ± 6.96g; 3rd weighing = 3.27 ± 10.19 g). The weight of *P. plantaginis* pupa is not affected by a prophylactic fumagillin treatment of the larvae before overwintering (Table 2: mean pupa weight: control = 198.03 ± 48.97 g; fumagillin = 203.06 ± 49.96 g), although male pupae are heavier than female pupae (Table 2: mean pupa weight: males = 181.89 ± 35.62 g; females = 236.56 ± 52.61 g).

Effect of fumagillin on egg laying- and hatching success. Egg laying success was evaluated by comparing the pairings that did not lay eggs (0 eggs produced 3 d post mating date) as well as the pairs that successfully laid eggs (at least one egg laid 3 d post mating date). We

Table 2. Effect of fumagillin on weight of *P. plantaginis* larvae and pupa

Source of variation	df	MS	*F*	*P*
Weight				
larva				
treatment	1	44.59	0.550	0.458
day	1	69.33	0.856	0.355
treatment:day	1	10.96	0.135	0.713
residuals	1,162	81.01		
pupa				
treatment	1	0	0.000	0.997
day	1	156,974	83.159	<2e–16
treatment:day	1	2,616	1.386	0.240
residuals	213	1,888		

Results of ANOVA testing for the effect of treatment on larva weight as well as pupa weigh. Analysis for larva weight also use day (before and after hibernation) and the interaction between treatment and day as factors. The ANOVA testing for the effect of treatment on pupa weight also tests for effect of gender and the interaction between treatment and gender (df = degrees of freedom; MS = Mean Square, *F* = *F*-value; *P* = significance probability).

found no significant effect of antibiotic treatment on egg laying success (Fig. 3a: Zero-inflated Count Data Regression: estimate = −0.405, z-value = −0.561, Pr(>|z|) = 0.575), but mating pairs treated with the antibiotic laid less eggs than control mating pairs (Fig. 3b: Zero-inflated Count Data Regression: z-value = 236.13, Pr(>|z|) = <2e-16). The proportion of hatched larvae per number of laid eggs is not significantly different between the control and antibiotic treatment groups, even though hatching success is higher in the antibiotic treated group (Fig. 3c: Generalized Linear Mixed Model via PQL: *P* = 0.246 and Fig. 3d: Linear mixed effects model: t-value = 0.357).

Discussion

We found significant effects of fumagillin treatment on central life-history traits in wood tiger moths but at the same time antibiotic treatment did not affect either larval overwintering survival or weight gain. Antibiotics are commonly added to artificial diets of mass-reared insects to suppress diseases and infections, which can easily spread in laboratory-reared colonies. However, there is limited knowledge on how the antibiotic itself might influence important life-history traits like overwintering performance and reproductive success. The observed negative effects in our experiment are related to reproduction and immune responses, and are important both in lab and wild insect populations. The lack of negative effects on larval survival can however be misleading to users of antibiotics in insect mass-rearing and skew the population fitness characteristics.

Guaranteeing a good overwintering performance is an important factor for mass-rearing techniques to maintain a functioning insect population. Overwintering is a highly sensitive life stage, affected by various factors, and can greatly influence the insects' quality (Bale and Hayward 2010, Xu et al. 2011, Spurgeon 2012). Our results show that the antibiotic treatment does not affect the larval overwintering success. Survival and weight gain were also similar between treated and nontreated individuals. It is known that antibiotics can interact with intestinal microbiota, resulting in growth promoting effects by changing the gut flora, and thus promote better weight gain (Lin 2011). We did not see a growth promoting effect, initiated by fumagillin, on larval weight gain performance, but in this study it remains unanswered, whether this is caused by the antibiotic not affecting the insects' microbiota. Thus, the 4-wk continuous fumagillin treatment seems not to affect the larval ability to prepare for hibernation. A prophylactic antibiotic treatment might help to limit the risk of infection without affecting the larval ability to overwinter.

While previous studies have focused only on how fumagillin is affecting infected insects, we examined the possible negative effect of the antibiotic on the lifespan development of noninfected lepidopteran larvae. A study with the mosquito *Anopheles stephensi* has revealed possible negative effects of the toxin fumagillin on insects; the larval developmental period was prolonged, rising with increasing antibiotic dose (Rutledge 1970). Our results did not display a similar effect on noninfected Lepidoptera, as the development of *P. plantaginis* larvae was actually shortened by the fumagillin treatment, whereas the overall developmental time from egg to adult was neither shortened nor prolonged. A prophylactic antibiotic treatment in mass-reared colonies could thus be used without expecting negative effects on the overall insects' development, even in the absence of a disease.

Fumagillin treatment increased the immune response of larvae, measured as activity of phenoloxidase in the hemolymph, meaning that the antibiotic affects immune- related enzymes. Antibiotics might be recognized as nonself by the insects' immune system. Consequently, the immune response will be up-regulated, resulting in increased enzyme levels. On the other hand, Fallon et al. (2011) found a limited hemocyte activity in hemolymph of *Galleria mellonella* larvae treated with fumagillin (Fallon et al. 2011). This showed, that the ability to fight infections decreases after being exposed to the antibiotic, resulting in a lower survival. A treatment of not obviously infected or sick individuals does not reveal the same results, as the survival was not affected and at the same time the immune response was activated. Thus, we propose that the antibiotic is recognized as nonself by the immune system. Because up-regulation of immune response is related with high costs as well as with the production of reactive oxygen species, this can result in a decrease of other fitness traits, especially when exposed to an infection.

Our study shows a significantly reduced number of laid eggs by fumagillin treated adults. These results, together with those from studies on the effect of fumagillin on *Nosema* infections in *Bombyx occidentalis* (Whittington and Winston 2003) and *A. mellifera* (Webster 1994), reveal negative consequences on brood levels, indicating that antibiotics negatively affect insects' reproduction success. Establishing new wild populations as a lab colony, however, implies mainly the maintenance of the insects' reproductive success. It is known that maternal stress can affect offspring quality by altering fitness traits such as hatching success, growth, or development (Mousseau et al. 1991, Kyneb and Toft 2006) as shown for instance in rove beetles (Kyneb and Toft 2006) and *Trichoplusia ni* larvae (Freitak et al. 2009). Offspring quantity and quality could also trade-off, such that large egg clutches might result in smaller offspring, whereas the hatching success and fitness of larvae from smaller egg clutches might be higher (Koch and Meunier 2014). Interestingly, the antibiotic treatment decreased the number of laid eggs while slightly increasing the number of hatched larvae. Still the proportion of hatched larvae per eggs laid did not differ between treatments. A prophylactic fumagillin treatment does, however, limit reproductive success. How this might affect long-term fitness, measured as decreased population size in the following generations, remains unanswered.

Our findings offer a better, more holistic understanding of prophylactic medicine treatments for laboratory-reared Lepidoptera

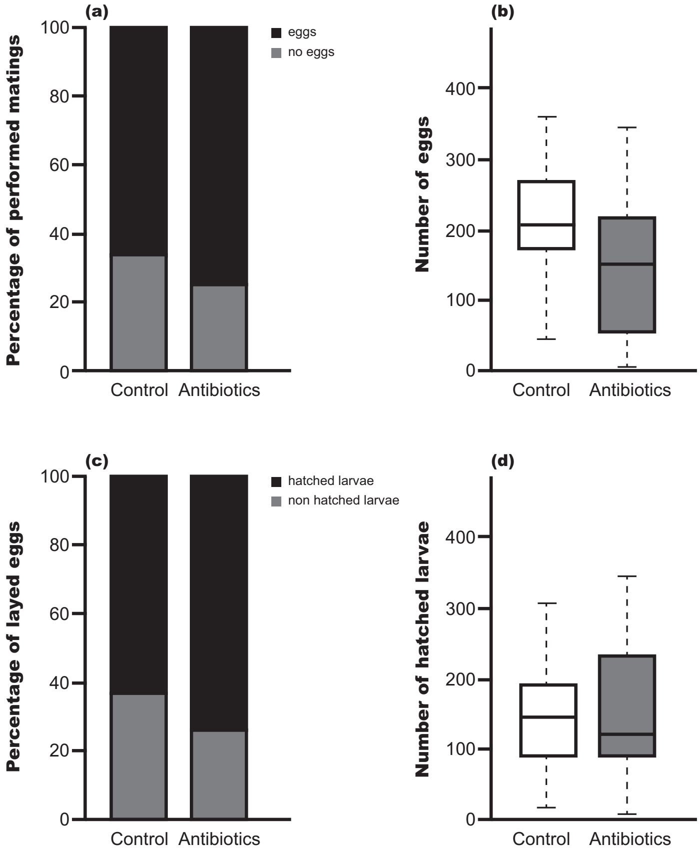

Fig. 3. Effect of fumagillin on egg laying and hatching success in *P. plantaginis*. Egg laying success was evaluated by (a) presence/absence of eggs 3 d post mating for control and antibiotic treated larvae; and (b) amount of laid eggs, counted 3 d post mating for control and antibiotic treated larvae. Hatching success was evaluated by (c) hatched and nonhatched larvae in proportion to the amount of laid eggs and also as (d) total number of hatched larvae, counted 21 d post egg laying date for control and antibiotic treated larvae.

larvae. Even though the application of antibiotics as prophylaxis is a common strategy in mass-rearing of insects, there was no recent study about possibly negative side effects. In conclusion, we show that although a prophylactic fumagillin treatment does not adversely affect larval development, it has a negative effect on reproductive success. The application of antibiotics in mass-reared insect colonies should thus be carefully considered, and possible negative side effects taken into account.

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Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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