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Liam Murphy

# Defensive Symbiosis of the Wood Tiger Moth (*Arctia plantaginis*)

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
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# ABSTRACT

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Microbial contributions to the protection of insects can impact on a host's fitness but the dynamics of these symbioses can vary more than many nutritional-symbiont associations seen in the literature. The wood tiger moth (*Arctia plantaginis*) secretes defensive fluids when attacked by avian predators, and they are home to bacterial communities including genera known to synthesise compounds similar to those found in the secretions. I studied the role bacteria play in the efficacy of the defensive secretions against avian predators and their contributions to the pyrazine chemical components of the secretions. I characterised the spatial and temporal variability of the secretion's bacterial taxa, and the impact on the life histories of *A. plantaginis* following their depletion. The former was done by manipulating the microbiome with antibiotics and testing the subsequent defensive secretions with predator assays and GC/MS. The latter used sequencing of the 16s rRNA gene to identify the bacteria in defensive secretions from wild moths, while life history traits of *A. plantaginis* were recorded with gene expression data following antibiotic treatment of larvae. Bacteria-depleted secretions did not illicit hesitation from birds in the predator assays, but the birds' perception of the secretion's taste remained unchanged. Chemical analysis showed no changes in the secretion's methoxypyrazine concentrations. Analysis of the microbiome revealed that bacterial taxa remained similar across a wide geographic area and multiple genetic populations of A. plantaginis, but there were significant changes in the microbiome composition over time. Following depletion of bacteria, *A. plantaginis* up-regulated their growth related genes and down-regulated immune system genes. They reached adulthood sooner, while adult females were significantly lighter without any loss in fecundity. The bacteria are contributing to olfactory cues directed towards avian predators, but it is not one of the prominent methoxypyrazines meaning further relevant compounds are present in the defensive secretions that have not been identified yet. The loosely associated bacterial taxa may form a functional core in which multiple taxa can contribute to the secretion's efficacy. The need for the immune system in controlling bacteria in the moth's body is costly for the host.

Keywords: Bacteria; chemical defences; microbiome.

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# TIIVISTELMÄ

Murphy, Liam
Puolustussymbioosi Täpläsiilikäsissä (*Arctia plantaginis*)
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Mikrobit voivat monin eri tavoin vaikuttaa isäntäeläintensä kelpoisuuteen ja isäntäsymbionttien dynamiikkaan. Täpläsiilikäs (*Arctia plantaginis*) erittää niskassaan olevasta valerauhasesta puolustusnesteitä, jotka sisältävät kemiallisia yhdisteitä, kuten metoksipyratsiineja. Nämä yhdisteet ovat tehokkaita torjumaan lintujen saalistusta. Puolustusnesteissä elää myös monilajinen mikrobiyhteisö. Osan näistä mikrobeista tiedetään syntetisoivan metoksipyratsiiniyhdisteitä. Väitöskirjassani tutkin bakteerien roolia puolustuseritteiden tehokkuudessa lintupetoja vastaan ja niiden merkitystä puolustusnesteiden pyratsiiniyhdisteiden muodostumisessa. Tutkin eritteissä esiintyvien bakteerien alueellista ja ajallista vaihtelua. Lisäksi manipuloin mikrobiomia antibiooteilla ja testasin puolustusnesteiden tehokkuutta lintuja vastaan. Bakteerien tunnistamiseen käytin 16s rRNA-sekvensointitekniikkaa. Antibioottikäsittelyn jälkeen analysoin kaasu- ja nestekromatografiaa käyttäen metoksipyratsiinien määrää puolustusnesteissä sekä tutkin muutoksia täpläsiilikkään elinkiertopiireissä ja käsittelyn vaikutusta tärkeiden elinkiertopiirteiden geeniekspressioon. Antibioottikäsittely vähensi merkitsevästi puolustusnesteiden bakteerien määrää ja diversiteettiä, mutta se ei vaikuttanut puolustusnesteiden metoksipyratsiinien määrään. Kemialliset analyysit eivät osoittaneet muutoksia eritteiden metoksipyratsiinipitoisuuksissa. Linnut kuitenkin epäröivät pidempään hyökätessään kontrollisaaliiseen (joita ei oltu käsitelty antibiootilla, mutta puolustuseritteillä). Antibioottikäsitellyillä perhosilla niiden immuniteettia säätelevät geenit olivat alentuneita. Antibioottikäsitellyt naaraat kasvoivat nopeammin, mutta olivat kooltaan pienempiä kuin kontrollinaaraat. Puolustusnesteissä olevat bakteeriyhteisöt ovat hyvin samanlaisia maantieteellisesti laajalla alueella eivätkä korreloi isäntäperhosen populaatioiden geneettisen rakenteen kanssa. Sen sijaan puolustusnesteiden mikrobiomin koostumuksessa tapahtui merkittäviä muutoksia ajan myötä. Tutkimukseni osoittaa, että bakteerit vaikuttavat puolustusnesteiden hajuvihjeisiin, mutta eivät tee sitä metoksipyratsiinien kautta. Tämä tarkoittaa, että puolustuseritteissä on todennäköisesti muita petopuolustuksen kannalta tärkeitä yhdisteitä, joita ei ole vielä tunnistettu. Nämä löyhät mikrobiassosiaatiot perhosen ja mikrobien välillä muodostavat eräänlaisen ”toiminnallisen ytimen”, jossa useat mikrobitaksonit voivat edistää puolustusnesteiden tehokkuutta. Bakteerien kontrollointi immuunijärjestelmän kautta on kuitenkin kallista isännälle.
Avainsanat: Bakteerit; kemiallinen suojaus; mikrobiomi.
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# CONTENTS

## 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.** Murphy, L., Mappes, J., Nissinen, R., Burdfield-Steel, E., Rojas, B., Weiss, B., Kaltenpoth, M., & Galarza, J. 2022. Contribution of the microbiome to the efficacy of defensive secretions of the wood tiger moth (*Arctia plantaginis*). Manuscript.

**II.** Murphy, L., Mappes, J., Galarza, J. A. 2022. Impact of host population structure on bacterial associates in the defensive secretions of the wood tiger moth (*Arctia plantaginis*). Manuscript.

**III.** Murphy, L., Mappes, J., Galarza, J. A. 2022. Some Things Never Change: But Does the Microbiome of the Wood Tiger Moth? Manuscript.

**IV.** Galarza, J. A., Murphy, L., Mappes, J. 2021. Antibiotics accelerate growth at the expense of immunity. *Proceedings of the Royal Society B*, 288, 20211819.

Table of author contribution to the original publications.

| **Study** | I | II | III | IV |
|---|---|---|---|---|
| **Original Idea** | JG, JM, EB, BR | JG, JM | JG, LM | JG, JM, LM |
| **Study Design** | JG, JM, EB, BR, LM, RN, MK, BW | JG, JM, LM | JG, LM, JM | JG, JM, LM |
| **Data analysis** | LM, JG | LM, JG | LM, JG | JG, LM |
| **Writing** | LM, JG, JM | LM, JG | JG, LM | JG, LM, JM |

JG = Juan Galarza, JM = Johanna Mappes, LM = Liam Murphy, EB = Emily Burdfield-Steel, BR = Bibiana Rojas, RN = Riitta Nissinen, MK = Martin Kaltenpoth, BW = Benjamin Weiss

# 1 INTRODUCTION

## 1.1 Microbiomes

Studies into the associations between bacteria and multicellular host organisms have increased in scope and breadth as sequencing technology and bacterial culture techniques have developed in the 21st century (Cullen *et al.* 2020). Bacteria present in the digestive tract have garnered the majority of research into host-symbiotic bacteria relationships, while in reality this is just one example of a microbiome in a host in which the bacteria have significant functional roles. Other microbiomes occur in a range of other body cavities, surfaces, within cells (Douglas 2015), and in haemolymph (Blow and Douglas 2019). Although bacterial communities and taxa have been characterised in these different microbiomes from ever increasing numbers of studied host species, the formation of these communities, their maintenance, and what functions they provide to the host are not always obvious or easily measurable (Haine 2007). A factor compounding the difficulty in designating the functions of a microbiome is the sheer level of variability of the microbiomes in different host species, meaning informed generalisations of the associations between host and microbiome are practically impossible to make. In the most tightly bonded associations, bacteria living within host cells, such as those seen in invertebrates, are crucial to host survival and vice versa (Nikoh *et al.* 2014). However, such associations contain very few species of bacteria, with even one bacterial taxon being normal in such a symbiotic relationship (Wernegreen 2012). These intracellular symbionts act similarly to mitochondria or chloroplasts, in that they over-produce specific resources that the host requires while being dependent on the host for many physiologically important biochemical pathways (Douglas 1997). At the other end of the spectrum are borderline interactions between bacteria and a host organism, in which a myriad of different bacterial taxa from the external environment may come into contact with the host accidentally, for example on foodstuffs the host consumes, before being emitted back into the environment

(Hammer *et al.* 2019). Such coincidental interactions rarely lead to bacteria performing direct functions for the host.

It is clear that the bacteria of microbiomes are not just involved in insular interactions with the host. They are modulating the hosts' interactions with the environment and neighbouring organisms and allowing different niches to be exploited by the host or the other actors involved in the interaction (Jones *et al.* 2017). Instead of being seen as bit players in ecosystem functioning or confined to nutrient cycling and disease, bacteria are key drivers at multiple levels of interaction between plants, herbivores and predators (Smith *et al*. 2015). While we increasingly see that microbiomes facilitate different functions and interactions, it can be more difficult to see common characteristics to the functional microbiomes across multiple study systems.

## 1.2 Characteristics of functional bacterial symbionts

### 1.2.1 Transmission

There are multiple mechanisms by which bacteria come to be present in a host organism's microbiome. These can be divided into two main groups: vertical transmission and horizontal transmission or acquisition. While the exact mechanism of bacterial transmission varies across different host-bacteria systems, vertically transmitted bacteria are passed directly from one host generation to the next generation. Horizontal transmission involves the recipient host obtaining the bacteria from its surrounding environment. Vertical transmission involves among the highest degrees of interdependence between the host and the bacterium (Wierz *et al.* 2021), especially if the bacterium is unable to exist or reproduce outside of the host and the host requires the symbiont for specific metabolic processes. Among the most widespread transmission mechanisms within vertical transmission is the incorporation of the bacteria in the oocytes of female known as transovarial transmission (McGraw *et al.* 2002). For example this is the mechanism observed in the widespread endosymbiont *Wolbachia* (Guo *et al.* 2018). Being included with the egg guarantees the transmission of the bacteria to the next generation but it can come at a cost for the host. To ensure their continued transmission within the host population, some endosymbionts can manipulate the host reproductive system (Osborne *et al.* 2009). Some cause complete mortality of male individuals or histoincompatibility between the gametes of infected and non-infected host gametes (Beckmann *et al.* 2019). An alternative vertical transmission strategy involves the host adults making the symbiotic bacteria available to offspring through various secretions. The female striped shield bug, *Graphosoma lineatum*, leaves secretions containing crucial symbiotic gut bacteria on the surface of eggs which the offspring consume once they hatch (Karamipour *et al.* 2016). Similarly, the beewolf, *Philanthus triangulum*, lines the walls of larval brood cells with secretions containing antibiotic producing Streptomyces bacteria which the larvae take up during their

development (Kaltenpoth *et al.* 2005). A final example of this is seen in the plataspid stinkbugs, which produce bacteria-filled capsids for their offspring to obtain their necessary bacterial associates after they have hatched (Kikuchi *et al*. 2011).

In the alternative horizontal mode of bacterial transmission, the bacteria can be acquired by the host in a number of different ways depending on the individual system being observed. In associations between marine organisms and their symbiotic bacteria, the bacteria can be present in the surrounding water and so is readily available to the host as seen in the bobtail squid system (Davidson *et al.* 2004). In terrestrial systems, bacteria can be obtained from the surrounding environment also (Flórez *et al*. 2015), although a major source of bacteria comes via the host's diet (Yun *et al.* 2014). As a result, microbiomes comprised of environmentally-sourced bacteria can contain multiple strains or phylotypes of bacteria (Douglas 2015). With greater structural complexity of the organ(s) in which the microbiome is situated, these microbial communities can contain up to thousands of different bacteria even. As a result, some of the bacteria can be entirely commensal, with no strong connection to the host (Hammer *et al*. 2019). However, there are examples of tightly-linked relationships in which horizontally obtained bacteria are very important in the development of the host. A prime example of this is the stinkbug, *Riptortus clavatus*, which obtains its *Burkholderia* symbiont from the environment each generation (Kikuchi *et al.* 2007). Without the presence of the symbiont in crypt cells lining part of the gut, the adult stinkbugs emerge with significantly stunted growth and weight. Such specific symbioses involving horizontal transmission of symbionts are considered exceptions to the general rule due to the increased inherent risk of not acquiring a crucial piece of a metabolic pathway in subsequent generations which can be minimised in vertical transmission (Clay 2014).

When viewed in the context of their functional importance, many terrestrial, insect-associated symbiotic bacteria that are involved in the synthesis of physiologically important compounds or defensive chemicals are vertically transmitted to each host generation (Clay 2014, Flórez *et al.* 2015). However, we know that there are exceptions to this generalisation. So other ecological characteristics of functionally important bacteria and their hosts must be taken into account.

### 1.2.2 Prevalence and community structure

A defining characteristic of symbiotic bacteria is how abundant they are in the host population. In the case of obligate symbionts which the host must have to survive, the symbiont is present across the entire host population (Kucuk 2020). In the case of endosymbionts which are not necessarily needed by the host such as *Wolbachia* (facultative symbionts), they can vary in their prevalence within the host population (Osbourne *et al*. 2009) and across different host populations (Meriweather *et al.* 2013). The same general rule applies for extracellular symbiotic bacteria as seen in the multitudes of studies examining the gut microbiomes of various insect species. For example in the stinkbug symbiosis

system, the mutualistic *Burkholderia* gut bacteria are present in all host individuals (Kikuchi *et al.* 2011). However, in systems where the gut bacteria not generally thought to provide crucial services to the host, associations between the host and the bacteria range from weak to even non-existent (Hammer *et al*. 2019). A well described case exhibiting this lack of linkage between host and bacteria come from the gut microbiome of Lepidoptera, in which no bacterial taxa are known to be needed for host functioning or development (Voirol *et al.* 2018). As a result, very few bacterial taxa are present in the gut microbiomes at consistent level across the sampled host Lepidopteran populations (Hammer *et al.* 2017).

Further insight into insect-bacteria associations can also be made through the characterisation of the bacterial communities present. In a similar vein to the prevalence of important bacterial associates in the host population, the level of complexity in the microbiome composition can also be informative. In many microbiome systems, obligate associations can be characterised by the lack of diversity in the bacteria present. For example, the microbiome of bed bugs (*Cimex lectularius*) is dominated by two bacterial taxa, which account for 97% of all bacteria in the host bed bugs (Meriweather *et al.* 2013). The gut microbiome of the Heteropterans, *Riptortus clavatus* and *Leptocorisa chinensis*, are home to just a single bacterial taxon in the majority of individuals (Kikuchi *et al*. 2005). In both of these cases, the bacteria have been shown to be obligatory for maintaining the hosts' fitness. Alternatively, some microbiomes can contain many different bacterial taxa, of which only a small number are providing services for the host. In the harlequin ladybird (*Harmonia axyridis*), the gut microbiome is home to a relatively diverse community of bacteria, however only two taxa from *Serratia* and *Lactococcus* are present in all sampled individuals are thought to provide the host with chemicals used to defend the beetle (Schmidtberg *et al.* 2019).

## 1.3 Symbiont derived defences

A particularly impactful function microbiomes can provide is the defence of the host from external threats including parasites, parasitoids, pathogens, and predators (Flórez *et al.* 2015). The bacteria can carry out these functions in a variety of ways including exclusion of pathogens (Gupta and Nair 2020), priming the host immune system (Anselme *et al.* 2008), and producing chemical defences (Smith *et al*. 2015). The latter has been recorded in multiple systems, especially in metazoans in marine environments (Flórez *et al*. 2015). However, research on anti-predator defences mediated by bacteria in insects is less apparent and difficult to directly prove in many cases (Clay 2014). There have been attempts to generalise the conditions under which defensive microbiomes in insects occur. Flórez *et al.* (2015) claim that the majority of defence mediating bacteria are horizontally acquired taxa, that bacterial taxa more readily colonise surfaces on the host as opposed to specialised cells, and in most systems, bacteria do not infect the entire host population. However, there are growing numbers of

exceptions to these three generalised characteristics. In the case of the Asian citrus psyllid (*Diaphorina citri*), the bacteria involved in defence is an obligate symbiont that is maintained inside bacteriocytes and vertically transmitted to offspring (Nakabachi *et al.* 2013). The endosymbiotic bacteria of *Paederus sabaeus* is also maintained inside specialised cells as opposed to surfaces of host tissues, although this system conforms to the generalisation in its facultative nature for the host (Kellner 2002). And as referenced in the previous section, horizontally acquired bacterial associates are present in all sampled individuals in the harlequin ladybird defence symbiosis (Schmidtberg *et al.* 2019). While Flórez *et al.*'s generalisations can match some defensive symbioses, there are many exceptions also. Nonetheless, symbiotic bacteria would not be maintained in a host population at all unless they are providing a fitness advantage for the host (Brownlie and Johnson 2009). Taking into account the range of associations requires researchers to fully screen each insect-bacteria defensive symbiosis on their own individual dynamics. This is necessary if we wish to get a proper understanding of them, including how they are formed, the level of protection they afford the host, and if there are any costs associated with them.

## 1.4 Host control of the microbiome

One striking characteristic of defensive symbioses in the literature is the fact that many of them are facultative in nature (Haine 2007). If the protection afforded by the bacteria to the host was crucial in maintaining the host's fitness then the bacteria would be constantly present in their respective hosts' microbiome as is the case in many nutritional symbionts (Wernegreen 2012). There must be a cost involved in having a microbiome and as a result conditions in which it is potentially positive for the host's fitness to be uninfected with symbiotic bacteria. The two obvious avenues for host costs to be accrued are in the provisioning of resources to the microbiome itself, and maintaining immune system functioning to control the microbiome's contents and position (Gupta and Nair 2020). This is not an indiscriminate use of the immune system towards all bacteria, rather a non-random acceptance of specific taxa (Scheuring and Yu 2012). It has been experimentally shown that insects can differentially express immune responses towards beneficial and pathogenic bacteria (Mikonranta *et al.* 2014), but they must still maintain control over the microbiome lest it become opportunistic and increasingly detrimental to the host (Foster *et al.* 2017). Among the most important immune functions insects use to control bacteria in their body is through the production of antimicrobial peptides (AMPs). Experimentally, AMP genes have been shown to target bacteria outside of specific bacteriomes in the weevil *Sitophilus zeamais* to keep its endosymbiont within the specialised tissue and stop its spread elsewhere in the weevil's body (Anselme *et al.* 2008) and the introduction of additional bacteria led to up-regulation of these AMP coding genes.

Bacteria in insect microbiomes can also come under intense pressure and bottlenecks due to the host's physiology and development. In holometabolous insects (insects that undergo complete metamorphosis to reach the adult life stage), the changes in internal morphology and chemistry during metamorphosis can lead to significant changes in microbiome content (Hammer *et al.* 2014). Many bacteria are unable to survive such changes and in some cases it gives host insects a chance to uncouple symbiotic relationships with bacteria before reaching adulthood (Hammer and Moran 2019). Finally, resident bacteria in the microbiome can play a role in the maintenance of the host's microbiome through the exclusion of potentially pathogenic microbes (Kucuk 2020). In some cases, symbiotic bacteria can even carry out this function for the host during metamorphosis, combining with host immune functions to manage the microbial community to the benefit and survival of the symbiont and host alike (Johnston and Rolff 2015).

## 1.5 *Arctia plantaginis* as a microbiome studies model species

The wood tiger moth (*Arctia plantaginis* (Rönkä *et al.* 2016)) is an emerging model species which has been used in studies on aposematism (Rönkä *et al.* 2020), polymorphism (Hegna *et al.* 2015), immunity (Nokelainen *et al.* 2014), and life history trade-offs (Lindstedt *et al.* 2020). Among the moth's many traits of that have garnered much attention are its defensive secretions that are emitted from the prothoracic glands. Despite their name, these are not true glands, with microscopy showing that they are melanised invaginations on the anterior end of the thorax (Fig. 1). The secretions are an ecologically important trait that are released when the moth is attacked by avian predators (Rojas *et al.* 2017). The secretions contain a number of chemicals such as pyrrolizidine alkaloids (Winters *et al.* 2021), but the primary olfactory components found to deter birds are the methoxypyrazines, 2-isobutyl-3-methoxypyrazine (IBMP) alongside 2-sec-butyl-3-methoxypyrazine (SBMP) (Rojas *et al.* 2017). The pyrazines are produced de novo in the moth, with diet manipulation experiments showing that they are still produced when the larval diet does not contain such chemicals (Burdfield-Steel *et al.* 2018). The adults themselves are capital breeders (Ojala *et al.* 2005), and thus do not feed at all and have no access to chemicals from the external environment, relying solely on what has been collected and built up as larvae to maintain their short adult life. Similarly, this trait also limits the possibility of bacteria invading the adult moths following eclosion. These traits lead to the defensive secretions being a more 'closed' microbiome system compared to generally transient gut microbiomes of other Lepidoptera (Hammer *et al.* 2017), and less likely to suffer acute fluctuations in the microbiomes constituent bacterial taxa.

Preliminary investigations discovered the presence of many bacteria in the defensive secretions of *A. plantaginis*. Among the taxa found were a number of bacterial genera, including *Pseudomonas* and *Staphylococcus* that are known to synthesise methoxypyrazines under certain conditions. This co-occurrence of

bacteria and methoxypyrazines opened the possibility that there is a contribution to the moth's chemical defences from the bacteria. Outside of the potential role of chemical contribution, how the bacterial community is formed in the defensive secretions and how fixed the composition of the bacterial community is remains to be seen.

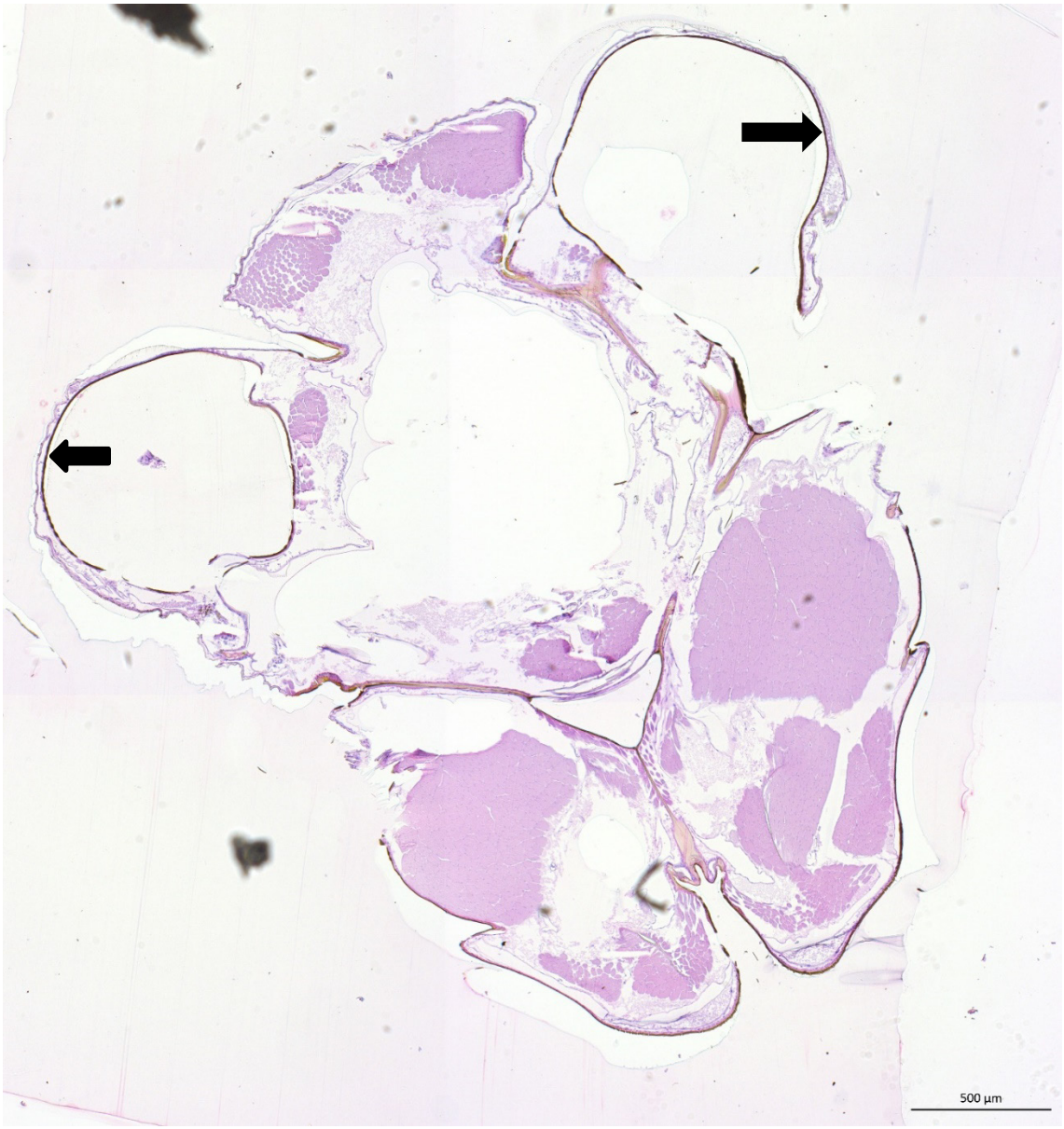


Figure 1. Cross section of the thorax of *A. plantaginis*. Note the two prothoracic glands on the dorsal side displaying melanised surfaces and a lack of specialised gland cells surrounding the cavity or invagination denoted by the black arrows.

## 1.6 Aims of the thesis

While there is an increasing body of knowledge on the dynamics of microbiome-insect host relationships and their contributions, many systems are represented by superficial data sets. Outside of economically important insects and model species, other species have been screened for their associated microbes in limited studies and most have not been characterized at all. In this thesis, I aim to characterize the bacterial community present in the defensive secretions of *A. plantaginis* both spatially and temporally in wild populations, alongside community data collected from lab-reared moths. Building up a robust overview of the microbiomes constituents, their variability, and their prevalence should allow for the most informed hypotheses on the assembly of the microbiome, the sources of the bacteria, and point to which bacteria hold the most potential importance for the host moths.

I also want to develop an understanding of the microbiome's contribution to the defensive secretions and their efficacy in deterring attacks from predators. To get a biologically relevant understanding of the microbiome's contribution, I must include both results from predators' reactions to the moth's secretions alongside quantifying the active components in the secretions.

Finally, I aim to discover if there are costs for the moths associated with maintaining their microbiome and general immune functioning, and link the impact of these processes with the moth's life history traits and fitness. This would require manipulation of the moth's microbiome during development.

# 2 METHODS

## 2.1 Larval rearing

For the experiments in I and IV, F1 generation larvae from moths caught in Tvärminne, Southern Finland were reared in the laboratory. Larvae were separated in to family groups of 12 larvae and maintained in ventilated, plastic containers. These were fed an artificial diet (containing distilled water, polenta, wheat germ, agar, and Vanderzant vitamin mix for insects (SigmaAldrich)) and predominately raised in climate cabinets (18:6 light-dark cycle).

Antibiotics for the larvae were prepared based on the methods of Zha *et al*. (2014). Preliminary tests were done to ascertain what dosage of antibiotic the larvae could withstand without negative effects on the groups survival during development. The dosages tested included 1 mg ml−1, 0.5 mg ml−1 and 0.01 mg ml-1. Following the preliminary tests, a final dosage of 0.04 mg ml-1 was decided upon as a safe sub-therapeutic concentration to use. Tetracycline and ciprofloxacin were dissolved in double distilled water. At the third instar, larvae from the treatment group received an injection of 1µl of the antibiotics solution into the third last segment of their body. During the fourth and fifth instar, the same larvae received a further injection of 2 and 3 µl of antibiotic solution respectively. Larvae in the control group were pierced by the microneedle in the same location at the same development stages to control for the wounding effects that could occur.

The life history traits of both groups were recorded during their development and adult life stages. The length of time until pupation occurred, time spent as pupa, and the survival were recorded in the earlier life stages. Adult females from both groups were either weighed 24 hours after being frozen, or had their abdomen dissected and the number of eggs in the abdomen counted to accurately reveal their fecundity.

## 2.2 Microbiome Characterisation and Gene Expression

Three methods of DNA preparation and sequencing were used in the course of the four studies. In chapter I, Sanger sequencing was used for the sequencing and identification of the cultured bacterial isolates. In chapters II and III, to assess the bacterial diversity in the defensive neck fluids we amplified ~1550bp of the gene coding for the 16S ribosomal RNA (rRNA) subunit. I and IV used sequences of the V1-V2 region of the 16s rRNA gene that had been sequenced using an IonTorrent PGM to characterise the bacterial community in the defence secretions. Custom sequencing primers for amplifying the V1–V2 region (approx. 350 bp of the DNA) were designed using Primer3 (Untergasser *et al.* 2012). Sequencing libraries were prepared following the instructions of the IonTorrent 316 chip amplicon preparation protocol. A synthetic bacterial mock community was included as a sequencing control (ZymoBIOMICS Microbial Community DNA Standard D6305) which included eight bacteria and two yeast species. In both sequencing methods, bacterial amplicon sequence variants (ASV) were determined using the R package DADA2 following the PacBio or IonTorrent pipeline (Callahan *et al.* 2019). Taxonomy was assigned to the ASVs according to the Silva database (Quast *et al.* 2013).

24 hours after receiving the antibiotic doses, a larva from each family was taken for RNA extraction. 24 larvae representing both groups and treated instars were converted to 24 cDNA libraries according to Illumina's TruSeq mRNA-seq protocol. The libraries were sequenced using an Illumina NextSeq 500 sequencer. The subsequent reads were aligned to the *A. plantaginis* reference transcriptome to find which genes were expressed in each sample. Differential gene expression was calculated using the edgeR package (Robinson *et al.* 2010) in R. Genes had to display a log-fold difference of more than 2 to be considered significantly differentially expressed.

Gene expression validation was done via quantitative PCR using 4 candidate genes and 2 housekeeping genes as normalization controls. The relative difference in the expression of these genes was done with the delta Ct method (Schmittgen and Livak 2008) to see if the differential expression found here corroborated what was observed in the RNA-seq results.

## 2.3 Population Structure Analysis

Sampled *A. plantaginis* had their DNA extracted following sampling of their defensive secretions and their genomes compared to the *A. plantaginis* reference genome (Yen *et al.* 2020) and single-nucleotide polymorphisms (SNP) were found for each sample resulting in 12277 SNPs after quality control and filtering. Principal component analysis of the genome was carried out in PLINK v 1.9 (Chang *et al.* 2015) and this was visualized in R. Maximum likelihood estimations of the ancestry of each individual also used the SNPs, and numbers of genetic

clusters were set at 8 with cross validation set at 10 in Admixture v. 1.3 software (Alexsander *et al.* 2009).

## 2.4 Predation Assays

Behavioural assays of avian predator responses to the defensive secretions of *A. plantaginis* followed the methods outlined by Rojas *et al.* (2017) who designed behavioural experiments using *A. plantaginis* and blue tits (*Cyanistes caeruleus*). Avian predators were represented by blue tits with each blue tit being randomly assigned to one of three groups (bacteria-depleted, non-depleted, and handling group). The handling group was included to gauge baseline behaviour from the blue tits interacting with unmodified baits. The birds were trained to consume oat flakes prior to experimentation. Each bird participated in 4 trials. In trials 1 and 4, the birds were offered an oat soaked in distilled water to check if the birds were hungry before and after their trials. The flakes in trials 2 and 3 had been soaked in 15µl of diluted defensive secretions from a moth in the corresponding treatment group. In every trial, the amount of time the birds took to attack the oat flake and the proportion of the oat consumed were recorded alongside other behaviours such as dropping of the oat during handling by the birds. Each trial lasted either 5 minutes or until the oat was fully consumed within that time.

## 2.5 Chemical Analysis

I based the chemical analysis methods on those of Burdfield-Steel *et al.* (2018), which had been optimised for detecting pyrazines in *A. plantaginis*, with minor adjustments made to the programmed run of the gas-chromatography/ mass-spectrometry machine which was optimised for the detection of pyrazines. Volatiles in the samples were collected on solid phase microextraction fibres before being manually inserted into the machine. The oven started at 60℃ was ramped up to 260℃ over the course of 25 minutes. The ions 124, 138, and 151 were focused upon as they were representative ions for methoxypyrazines.

# 3 RESULTS

## 3.1 Characterisation of the microbiome

Culturing of bacteria ex-situ produced bacterial growth in all culturing plates containing the defensive secretions. Following re-plating and growth, 9 colony morphologies were characterized and sequencing of representatives from each colony morphology revealed 19 bacterial genera. Fluorescent in situ hybridisation revealed that no bacteria were present on the inner surface of the thoracic pseudo-glands of *A. plantaginis*. All bacteria were observed in the digestive tract and a large caecum located anteriorly to the digestive tract in the abdomen of the moth. Within the caecum, bacteria was concentrated more heavily around the edges of the organ.

Microbiomes and their constituent bacteria can be characterized through a range of methods involving culture dependent techniques and 16s rRNA gene sequencing. In II, we found a total of 956 ASVs, representing 65 genera, from 62 samples across a north-south 800km transect. All of the genera were found in each of the four sampling locations in Estonia, southern, central, and northern Finland. In the temporal characterisation, within southern Finland (III), a total of 813 ASVs were found from 87 sampled moth secretions. The bacterial taxa belonged to 90 known bacterial genera and 49 families. Within a single year, the α-diversity did not differ geographically, and there was no difference in β-diversity either between the four locations, with geographic location being very weakly correlated with the composition of the defensive secretion microbiome. The composition of the microbiome was not correlated with the genetic origin of host moths either. There were differences in the temporal study (III) though. Observed species richness did not differ over the four-year sampling period but α-diversity differed significantly between years. The β-diversity of the defensive secretions differed significantly across the four years.

The characterisation of the bacteria in the defensive secretions of laboratory raised A. plantaginis revealed the presence of 362 ASVs (I and IV), which is significantly less than what was observed from wild caught moths (II and III).

A single bacterial taxon, a member of the *Asinibacterium* genus, was present in over 80% of samples across the sampling locations and populations of *A. plantaginis* (II) and was the most abundant bacteria found across the four years sampled in southern Finland (III). *Asinibacterium*, was present in the majority of samples from the final sampling year, it was present in only a third of samples from the three years prior (III). Taxa from genera known to produce methoxypyrazines, *Staphylococcus* and *Streptococcus*, were among the ten most abundant taxa recorded in the study with relative abundances of 4-6%, but the majority of these observations came from the final year also. Other bacteria from known pyrazine producing families had relative abundances of less than 1% within the years they were observed.

## 3.2 Host Population Structure (II)

Analysis of the genetic structure of the *A. plantaginis* revealed very little variation between the moths from within Finland. Estonian *A. plantaginis* were clearly different to the Finnish population. Finnish samples could be differentiated based on the colour morph (yellow or white) that they represented. The white Finnish moths were still closer to the yellow morph from Finland than the distant white Estonian cluster. Finnish moths displayed a more mixed ancestry, whereas Estonians had high probability of shared ancestry within the same population bar two individuals that could represent immigration from Russian or Latvian population sources. There was no correlation between the genetic cluster the sampled moths originated from and the bacterial content of their defensive secretion.

## 3.3 Predation Assays (I)

The predation assay was carried out with 35 blue tits split across the three treatment groups (bacteria depleted = 14, non-depleted = 14, Handling group = 7). The proportion of the bait eaten by the blue tits was significantly lower if the baits contained any defensive secretions compared to the handling group's baits. However, there was no difference in consumption between baits containing secretions from bacteria-depleted and non-depleted group moths. The non-depleted baits caused the blue tits to hesitate and pause their attack significantly longer than baits containing secretions from the bacteria-depleted moths. Although the blue tits tended to drop the baits containing the non-depleted group secretions more than the bacteria depleted group, any difference observed was insignificant.

## 3.4 Chemical Analysis (I)

Although there were very high levels of variation in the amount of methoxypyrazines present in the defensive secretions of *A. plantaginis* in both treatment groups, overall there was no difference in the total amount of pyrazines present. There was no difference in the proportions of IBMP to SBMP within each sample between the two groups as well. Aside from naturally occurring individual variation, the depletion of bacteria in the defensive secretions had no impact on the content of methoxypyrazine compounds.

## 3.5 Gene Expression Analysis (IV)

I observed increasing numbers of growth-related genes, such as chitin and growth factors, being up-regulated, while immune function related genes, such as serpins, serine proteases and innate immune system genes, were increasingly down-regulated with each subsequent injection. Overall, 93 genes related to growth and immunity were differentially expressed following antibiotic treatment compared to untreated larvae in the control group. The most abundant of these were two genes related to growth and renewal of the larval exoskeleton (chitin binding and insect cuticle proteins). Following the very first injection of antibiotics, all genes that were differentially expressed were doing so via up-regulation. It was not until after the final injection that all but one growth related genes were up-regulated and all but a single immunity-related gene were down-regulated. It was also apparent that overall increased gene expression occurred in the late stages of larval development as seen in the overall gene expression profiles. The patterns in gene expression observed were confirmed by qPCR gene validation using candidate genes. The candidate genes were in agreement with the gene expression data in regard to genes being up or down-regulated.

## 3.6 Life history traits (IV)

Life history traits deviated significantly in the antibiotic treated group compared to those of the control group. Larvae from the antibiotic group reached pupation significantly quicker than the control group larvae. However, there was no difference in the amount of time either group spent as a pupae before eclosion occurred. Females belonging to the control group were significantly heavier as adults than those from the antibiotic group. This did not translate into differences in the number of eggs female moths contained. Survival of larvae in the antibiotic treated group was significantly higher than that of the control group. Compared to non-experimental stock, survival was lower than average levels of survival

observed, probably as a result of the invasive introduction of antibiotics via injection.

# 4 DISCUSSION/SYNTHESIS

The explorative characterisation of the defensive secretion microbiome of *A. plantaginis* produced a mix of complementary and contradictory results when viewed through the prism of previous microbiome characterisations in insects and Lepidoptera in particular. Within a single generation, a conserved, core bacterial community, centred on a member of the *Asinibacterium* genus, appeared to be widespread across a large geographic range of *A. plantaginis* in Finland and Estonia. This is very similar to the observations made on insect-bacteria associations that are very tightly linked and play functional roles for the host insect (Clay 2014). However, the temporal variability observed within the southern Finnish population suggests almost the opposite. There was little consistency or robustness in the bacterial community from year to year in moths living in the same area. The previously mentioned *Asinibacterium* was also far less prevalent in the three other years sampled compared to the year it was widely recorded across the geographic study. The lack of community stability in the defensive secretions was reminiscent of the gut microbiome characteristics noted in many Lepidoptera (Voirol *et al.* 2018). Such an apparently loose bacterial assemblage was also unlikely to harbour any functionally crucial bacterial phylotypes that would contribute substantially to the primary compounds needed for the defensive secretions to ward off avian predators. When viewed independently, these two sets of results can offer easy to interpret conclusions that correspond with results found in other systems in the microbiome literature. However, when the two studies are combined, conclusions cannot be so black and white. There could be widespread microbiome consistency in the defensive secretions across its north-eastern European range, but there is no sign of temporal stability. This leads me to wonder if the prevalent bacteria found in the defence secretions in any given year are more indicative of the environmental bacterial community rather than close associates of *A. plantaginis*. If this is the case, then the bacterial communities would be shaped more by prevailing weather conditions, vegetation growth, and soil conditions each year across Finland and Estonia. Bacteria in the moths must survive the host's immune system (Foster *et al.* 2017) and the physiological upheaval of metamorphosis

(Hammer and Moran 2019) to make their way to the defensive secretions. The bacteria are not completely random then, but a more select community that are resistant or tolerant of these potentially adverse conditions within the moths.

Regardless of their origin, the results of studies in I and IV show that the bacteria are not entirely passive hitchhikers in the moths and their secretions. The behavioural assays with a candidate avian predator, the blue tit, suggests that bacteria must be contributing to the olfactory cues given off by the defensive secretions. Although the bacteria only slowed the attack from the blue tits, in a natural interaction, greater predator hesitation would provide the moths with a higher chance of escaping before capture. Despite not forming a closer association with the moth as seen in many defensive symbioses (Flórez *et al*. 2015), the bacteria are nonetheless influencing a crucial ecological interaction for *A. plantaginis*. However, facilitating and maintaining the microbiome may come at a cost for the moths as seen in IV. Depletion of the bacteria present in the moths during development has significant effects on gene expression patterns and life history traits of the moth. The moths down-regulated multiple immune function genes following antibiotic treatment, redirecting their resources towards faster growth and development. While the exact mechanism behind these changes remains unclear, it sits in line with theoretical work pointing towards host organisms needing to invest in their immune system functions to control the microbiomes present in and on their bodies (Foster *et al.* 2017). Again, the bacteria recorded from the defensive secretions are linked to important host processes and recordable traits. Combining these results highlights the importance interactions with bacteria can have on their host, regardless of the bacteria's level of association (or lack thereof), and its ecology throughout its life.

Many questions remain to be answered following the studies carried out in my thesis. Namely, what further compounds derived from the bacteria are contributing to the defensive secretions efficacy and how abundant do the bacterial taxa have to be in the secretions to induce the functional changes to the secretions? However, the combination of thorough characterizing of the microbiome dynamics, chemical analysis, and ecologically relevant assays with other members of the moths' ecosystem lays the framework for other defensive symbioses to be examined in a similar, thorough manner. I predict that it would reveal numerous ecologically-meaningful interactions between microbes, hosts, and their predators

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# ORIGINAL PAPERS

# I

## CONTRIBUTION OF THE MICROBIOME TO THE EFFICACY OF DEFENSIVE SECRETIONS OF THE WOOD TIGER MOTH (*ARCTIA PLANTAGINIS*)

by

Liam Murphy, Johanna Mappes, Riitta Nissinen, Emily Burdfield-Steel, Bibiana Rojas, Benjamin Weiss, Martin Kaltenpoth & Juan A. Galarza 2022

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# II

# IMPACT OF HOST POPULATION STRUCTURE ON BACTERIAL ASSOCIATES IN THE DEFENSIVE SECRETIONS OF THE WOOD TIGER MOTH (*ARCTIA PLANTAGINIS*)

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# III

# SOME THINGS NEVER CHANGE: BUT DOES THE MICROBIOME OF THE WOOD TIGER MOTH?

by

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# IV

# ANTIBIOTICS ACCELERATE GROWTH AT THE EXPENSE OF IMMUNITY

by

Liam Murphy, Johanna Mappes & Juan A. Galarza 2021

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# Antibiotics accelerate growth at the expense of immunity

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Antibiotics have long been used in the raising of animals for agricultural, industrial or laboratory use. The use of subtherapeutic doses in diets of terrestrial and aquatic animals to promote growth is common and highly debated. Despite their vast application in animal husbandry, knowledge about the mechanisms behind growth promotion is minimal, particularly at the molecular level. Evidence from evolutionary research shows that immunocompetence is resource-limited, and hence expected to trade off with other resource-demanding processes, such as growth. Here, we ask if accelerated growth caused by antibiotics can be explained by genome-wide trade-offs between growth and costly immunocompetence. We explored this idea by injecting broad-spectrum antibiotics into wood tiger moth (*Arctia plantaginis*) larvae during development. We follow several life-history traits and analyse gene expression (RNA-seq) and bacterial (r16S) profiles. Moths treated with antibiotics show a substantial depletion of bacterial taxa, faster growth rate, a significant downregulation of genes involved in immunity and significant upregulation of growth-related genes. These results suggest that the presence of antibiotics may aid in up-keeping the immune system. Hence, by reducing the resource load of this costly process, bodily resources may be reallocated to other key processes such as growth.

## 1. Introduction

For the past 60 years, antibiotics have been widely used beyond the therapeutic treatment of disease, including pest control and growth promotion in a variety of taxa [1]. Worldwide, approximately 70% of all antimicrobials sold are used in animals intended for human consumption [2] . Although most research has focused on the role of antibiotics in terrestrial livestock, growth promotion through antibiotic supplementation has also been demonstrated in commercial aquaculture species [3]. In insect research, antibiotic usage has mainly focused on the treatment of bacterial infections [4], on the total or partial removal of endosymbiotic bacteria such as *Wolbachia* [5], and as potential pesticides towards some moth species [6].

In recent years, prophylactic antibiotics have been used in the raising and maintenance of large-scale insect cultures. With research laboratories aiming to maintain large numbers of insects, artificial diets and antibiotics are increasingly being used to make the rearing process more efficient. It has been realized that apart from limiting mortality due to infection, antibiotics can positively impact the growth rate of cultured insects. For instance, silkworms (*Bombyx mori*) treated with antibiotics have been found to grow to larger sizes and have heavier cocoons than untreated ones [4]. More recently, antibiotics were shown to promote the growth of several different insect species [7]. Thus, the growth-promoting effect of antibiotics seems to be common and not limited to vertebrates. Interestingly enough, there is a general lack of understanding about the molecular processes underpinning such an effect.

One way to deduce the possible mechanisms behind accelerated growth is by investigating changes in resource allocation. Because bodily resources are finite, as more resources are allocated to growth, fewer resources remain available for other processes. High resource-demanding processes are hence expected to be predominantly impacted by resource redistribution. Evidence from ecological immunity research shows that acquiring and maintaining immunocompetence is highly costly [8] and, as life-history theory suggests, expected to trade off with other important traits as a consequence [9]. This has been shown in several taxa including humans [10], insects [11], molluscs [12] and even plants [13]. Thus, we hypothesize that shifts in resource allocation between immunity and growth could potentially explain the accelerated growth observed across taxa. We investigate this hypothesis in an emerging insect model system, the wood tiger moth (*Arctia plantaginis*), through RNA-seq, 16S ribosomal profiling, as well as life-history analyses in the presence of antibiotics.

## 2. Material and methods

### (a) Study design

#### (i) Model species

The wood tiger moth (Erebidae), formerly *Parasemia plantaginis* [14], is an emerging model system with comprehensive genome resources available [15–18], and widely used in ecological and evolutionary research [19]. As adults don't feed, many factors during the larval stage, such as diet and their interaction with the environment, can have major effects later in life as adults [20,21].

#### (ii) Larval rearing

A split-family rearing design was implemented using the F1 from nine families. Each family was divided into maximum group sizes of 12 larvae, summing a total of 224 larvae each in control and antibiotic groups. The larvae were fed an artificial diet (electronic supplementary material, S1) replaced daily. Larvae in the treatment group were injected with two broad-spectrum antibiotics: tetracycline and ciprofloxacin once an instar. The larvae received three injections in total. The antibiotic solution consisted of 2 mg of tetracycline and 2 mg of ciprofloxacin dissolved into 100 ml of double distilled autoclaved water. The dose was determined by several trials using different concentrations (1 mg $ml^{-1}$, 0.5 mg $ml^{-1}$ and 0.01 mg $ml^{-1}$) following the methods of [5] until a subtherapeutic dose was obtained (0.04 mg $ml^{-1}$). The solution was injected using a sterile microneedle (10 µl) on the third last segment, parallel to the larval gut. The dose was increased by 1 µl in each subsequent injection. In parallel, larvae in the control group were pricked with an empty sterilized microneedle in the same location and for the same number of times as the treated larvae.

#### (iii) RNA-seq library construction

Twenty-four hours after antibiotics injection, one larva per family was taken for gene expression analyses and stored in RNA-later solution. A total of 24 pair-end (2 × 75 bp) cDNA libraries were constructed (four larvae/instar/treatment), according to Illumina's TruSeq mRNA-seq protocol and sequenced in an Illumina NextSeq 500 sequencer. The quality of the raw sequence reads was inspected with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After quality filtering and trimming, we obtained a mean of 18.2 million reads per sample, with a mean quality Phred score of 34.5 and a minimum length of 65 bp. All sequence data have been deposited in the National Center for Biotiechnology and Information (NCBI) under Bioproject PRJNA557336.

To calculate expression profiles, we aligned the high-quality filtered reads to the wood tiger moth's reference transcriptome [16]. We used the R package edgeR [22] to test for differential gene expression after each consecutive injection under a quantile-adjusted conditional maximum-likelihood (qCML) framework. Genes were considered to be significantly differentially expressed if they showed a log-fold difference of more than 2 and a $p$-value < 0.005 after a Benjamini and Hochberg correction for multiple testing [23].

We obtained a functional annotation of the expressed genes by blasting (BLASTx) [24] against a non-redundant protein database (nr) (NCBI; last accessed 06-05-2020). Gene ontology (GO) terms and information of protein family were obtained using InterProScan v. 72.0 [25]. The biological processes of the GO annotations were obtained using REVIGO [26]. A gene set enrichment analysis was performed with the R package topGO [27]. A Fisher statistic was computed using the *elim* algorithm to test for enrichment of biological processes according to the GO classification [28].

#### (iv) Gene expression validation

The RNA-seq expression profiles of a subset of four genes were validated through quantitative PCR (qPCR). We selected two genes involved in insect growth and two lipid transport genes that were up- and downregulated in all samples, respectively. As normalization controls (i.e. housekeeping genes), we selected one transcript from the RNA-seq data that showed a uniform expression level across all samples, and a gene (GADPH) known to have a stable expression in moth species in a variety of conditions [29]. The relative change in gene expression between the treatments was examined using the delta Ct method [30] taking into account multiple reference genes as described in [31].

### (b) Bacterial community analyses

To assess the effect that the antibiotic treatment had on the associated bacterial communities, we analysed the V1–V2 region of the ribosomal 16S gene sequenced in a IonTorrent PGM. Samples were taken from 42 adult males (21 per treatment) by gently squeezing their thorax with sterile tweezers to stimulate a defensive secretion from the back of their head. When attacked, adults perform a reflex bleed reaction to deter predators. Adults were chosen because they don't feed and thus taking up bacteria from the environment is unlikely at this life stage. Moreover, the secretion is an important survival trait for the species [32,33], and thus it is expected to be highly conserved, including its associated microbiota. The defensive secretion was collected under a laminar flow to minimize contamination using a sterile capillary and placed in a 1.5 ml Eppendorf tube containing 30 ul of autoclaved ddH20. DNA was extracted by inserting a metal bead (⌀ 2.3 mm) inside the Eppendorf tube containing the capillary and homogenized using a bead ruptor (OMNI). After homogenization, the samples were boiled at 110°C for 10 min in a Grant heat block and stored at −20°C until further use.

Custom sequencing primers (electronic supplementary material, table S5) for amplifying the V1–V2 region (approx. 350 bp) were designed using Primer3 [34]. Sequencing libraries were prepared following the instructions of the IonTorrent 316 chip amplicon preparation protocol. A synthetic bacterial mock community was included as a sequencing control (ZymoBIOMICS Microbial Community DNA Standard D6305) which included eight bacteria and two yeast species.

Bacterial amplicon sequence variants (ASV) were determined using the R package DADA2 following the IonTorrent pipeline [35]. Taxonomy was assigned to the ASVs according to the silva_nr99_v138.1_train_set [36]. Alpha diversity indices were obtained using phyloseq v. 1.36 [37]. We tested for differences

in the Chao1 and Shannon alpha diversity indices. The former provides estimates including estimations for unobserved taxa [38], whereas the latter considers the relative abundances of taxa and community evenness in its calculations [39]. One-way ANOVA followed by a Tukey's honestly significant difference (Tukey's HSD) *post hoc* test for pairwise comparisons were executed in R v. 4.1.0 [40]. Finally, differences in bacteria presence/absence and abundance (i.e. number of reads per taxa) between the treatments were estimated using the R package Metacoder v. 0.3.5 [41].

### (c) Life histories

We recorded the larval growth rate as the number of days from hatching until pupation, the number of days spent as pupa, the overall growth rate from larval hatching until adult eclosion, as well as the sex of the adults. Females from both treatment groups were weighed individually. Weight is commonly used as a proxy for fecundity as greater female body mass is linked to greater fecundity [42]. Additionally, we surgically extracted the eggs from the females' abdomen and counted them under a stereo microscope.

All of the analyses carried out for the life-history measurements were done in R (v. 3.5.0) [40] using the packages 'lme4' [43], 'coxme' [44] and 'MASS' [45]. Differences in growth rate were tested using a mixed-effect cox model setting the treatment group as a fixed factor and the sex as cofactor. Random factors included family and the eggs' lay date with family nested within the lay date factor as multiple families laid eggs on the same dates. Development time = treatment group + sex + (1/family/lay date).

The same formula was used when comparing time to pupation, time as a pupa and time to eclosion. Differences in mass and the mass accrued by larvae per day of development were compared using generalized mixed models following the same formula. Survival was tested by comparing survival curves by the Kaplan–Meier method and log-rank test as implemented in the R package 'survival' [46]. Differences in egg number were compared using a linear mixed-effect model which used the family as the random factor. Number of eggs = treatment group + (1|family). As count data often violates the assumptions of linear models (i.e. linearity, normality of residuals, homoscedasticity), we performed model diagnosis analyses consisting of Shapiro–Wilk normality tests and diagnostic plots to assess the suitability of the data to our model.

## 3. Results

### (a) Gene expression profiling

A total of 93 gene transcripts belonging to growth and immunity functional categories genes were found differentially expressed (logFC > 2, $p < 0.005$) (electronic supplementary material, table S1). The full annotation including analyses, accessions, and descriptions is provided in electronic supplementary material, table S2. The most abundant annotated differentially expressed gene transcripts were chitin binding (GO:0006030) and insect cuticle proteins (GO:0042302), both related to exoskeleton (cuticle) renewal. Likewise, we identified eight gene transcripts belonging to different growth factor classes: epidermal (EGF) 5 genes, Adenosine deaminase-related (ADGF) 2 genes, Tyrosine-protein kinases 1 gene, and also growth factor antagonists such as transforming growth factors (TGF) 2 genes. Congruently, the enrichment analysis indicated a significant enrichment (Fisher $p = 0.007$) of GO terms referring to chitin metabolic processes (GO:0006030) (electronic supplementary material, table S3 and figure S1).

As the number of antibiotic injections increased, we observed a trend in the upregulation of growth-related genes (chitin, growth factors) and a downregulation of immune-related genes such as serpins, serine proteases and innate immunity genes, hereafter immune genes. After the first antibiotic injection, most of the differentially expressed genes were upregulated. This was probably as a first reaction to the injection itself. The second injection, however, started the downregulation of immune genes and the upregulation of growth-related genes (figure 1). After the third antibiotic injection, all growth-related genes except one growth factor were upregulated, and all but one immune gene were found downregulated. The same pattern of more genes being expressed late in development was also observed in absolute expression profiles (i.e. not only in differentially expressed genes; electronic supplementary material, figure S4). The exception is the first antibiotic injection, which triggered substantial gene expression, probably due to the injection itself. Hence, it is unlikely that the differences observed in gene expression could be influenced by the natural change in gene expression as development occurs.

### (b) Gene expression validation

The qPCR validation for the candidate genes was in good agreement with the RNA-sequence data showing the same pattern of up- or downregulation in both datasets (electronic supplementary material, figure S2).

### (c) Bacterial community

A total of 362 ASVs were observed distributed across samples. In the synthetic mock community, only the expected bacterial species were recovered, indicating sufficient sequencing reads and no external contamination within the sequencing run. The ANOVA analysis showed a significantly lower ($p < 0.001$) diversity in both Chao1 and Simpson alpha diversity (figure 2). The *post hoc* test indicated significant differentiation between the antibiotics and control samples for both computed indices (electronic supplementary material, table S4).

A substantial reduction in bacterial taxa was observed in the antibiotic treatment including five full phyla removed, namely Acidobacteriota, Aquificota, Cyanobacteria, Fusobacteriota and Patescibacteria. This resulted in 13 families and 41 genera absent in the antibiotic treatment (figure 3; electronic supplementary material, figure S3).

### (d) Life-histories

The larval growth rate from egg hatching to pupation differed significantly (estimate = 0.0651, $p < 0.001$), with both sexes in the antibiotic treatment reaching pupation faster than controls (figure 4). There was no significant difference in the time spent as pupa (estimate = 0.0123, $p = 0.7057$). Hence, the significant difference (estimate = 0.0589, $p < 0.001$) in growth rate from egg hatching to adult eclosion for both sexes is solely due to the faster larval growth up until pupation (figure 4; electronic supplementary material, table S7).

Control females were significantly heavier than the antibiotic-treated females (estimate = 0.0812, $p = 0.0024$) (figure 4). This could be due to the shorter development time experienced by the antibiotic larvae (figure 4), which is suggested by a low ($R = 0.32$) but significant ($p = 0.027$) correlation between development time and weight of the

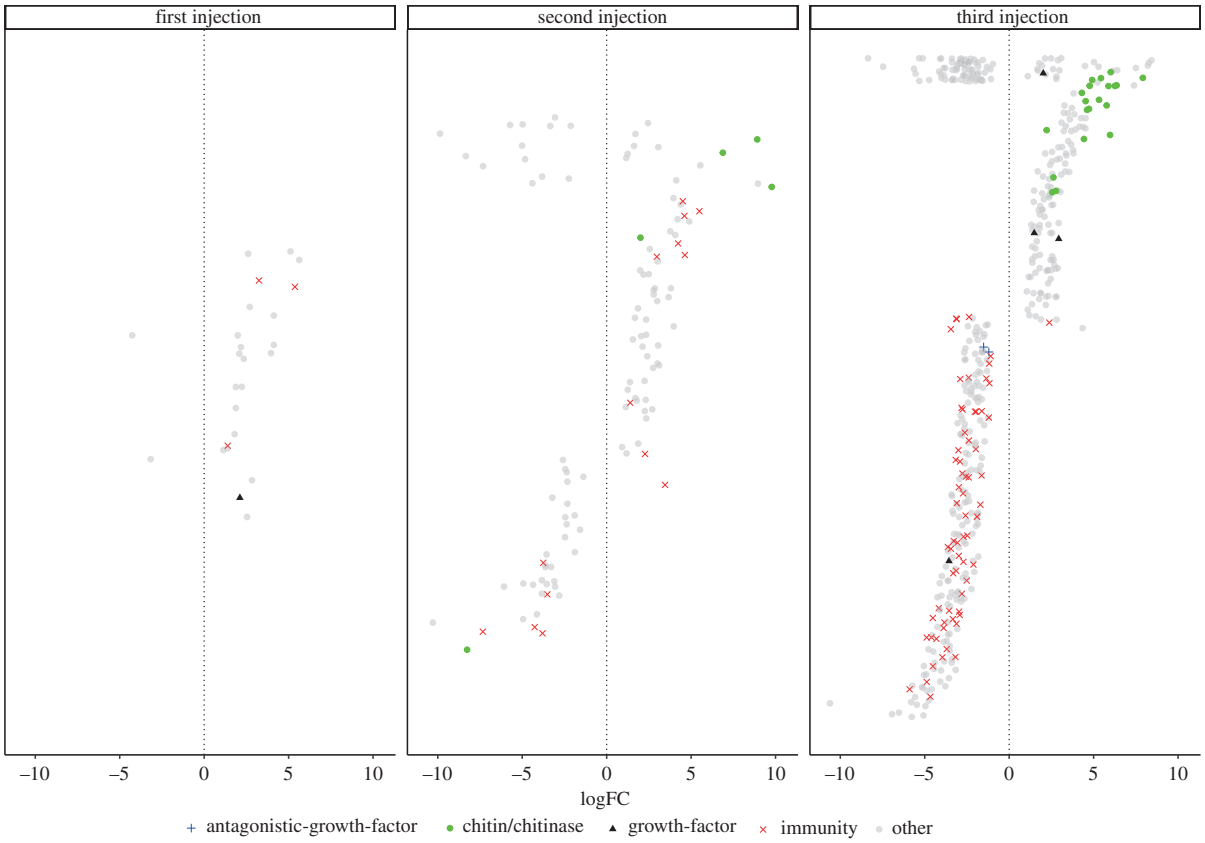


**Figure 1.** Expression patterns of growth and immune genes in the wood tiger moth (*Arctia plantaginis*) larvae after consecutive antibiotic injections. (Online version in colour.)

antibiotic-treated females (electronic supplementary material, figure S5A). The same pattern was observed between the number of eggs and female weight, which showed a strong correlation in the antibiotic treatment only (electronic supplementary material, figure S5B). However, we found no differences in the mean number of eggs (estimate 0.03308, $p = 0.2211$) after correcting for high-leverage values (figure 4; electronic supplementary material, figure S6). This suggests compensatory mechanisms for the smaller antibiotic-treated females in their reproductive output. The survival probability between the two treatments differed significantly (log-rank $p < 0.001$), particularly during the final larval instars (electronic supplementary material, figure S7). In laboratory conditions, larval mortality is typically 10–20% with the highest mortality peaks happening early and late in development. The mortality in our experiment was approximately 10% higher, most likely due to pricking itself.

## 4. Discussion

The use of antibiotics in animal husbandry has been widespread partly due to their positive effect on growth and mass gain. Nonetheless, the molecular mechanisms behind such an effect are still unclear. Here, using RNA-seq, r16S profiling and life-history analyses we investigated a potential trade-off between immunity and growth as a likely explanation. Our results suggest that the presence of antibiotics may aid in maintaining the immune system through a reduction of the bacterial load (i.e. total bacterial diversity and abundance). Hence, by reducing resources allocated for this costly process, bodily resources may be reallocated to other key processes such as growth.

### (a) Growth

Insect growth occurs through a series of exoskeleton (cuticle) renewals or moults. Moult succession includes the separation of the cuticle from the epidermis, or apolysis, and the synthesis of a new cuticle. Chitin is a main component of the cuticle of insects providing rigidity and articulation. Its turnover is regulated by two main enzymes, chitin synthase for its synthesis and chitinase for its degradation [47]. In this study, most of chitin, chitinase and cuticle protein genes were increasingly upregulated in the antibiotic treatment, suggesting an active cuticle turnover process. This may be a response in trying to keep up with an accelerated body growth indicated by the upregulation of growth factors and the downregulation of their transforming growth factors regulators. Congruently, other studies have demonstrated that insect growth factors play important roles in larval and pupal moulting, as well as in axon ingrowth and targeting [48,49].

### (b) Immunity

The innate immune system of insects consists of physical barriers such as the integument and the peritrophic membrane, as well as humoral and cellular responses [50]. When infected, haemocytes such as plasmatocytes and granulocytes transported by the haemolymph are activated leading to phagocytosis, nodule formation and encapsulation [51]. Invading microorganisms are recognized by pattern-recognition

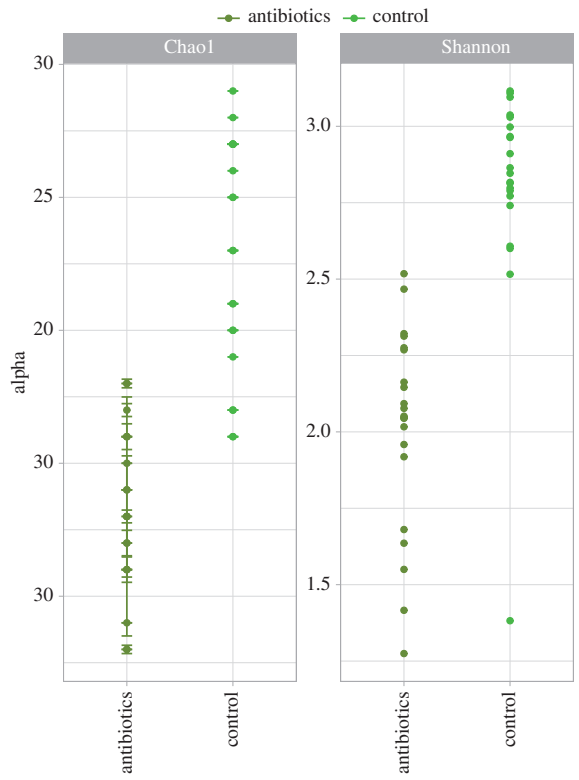


**Figure 2.** Bacterial alpha diversity indices associated with wood tiger moth (*Arctia plantaginis*) treated with antibiotics and untreated control. (Online version in colour.)

protein (PRPs) receptors that bind conserved domains located on the lipids and carbohydrates synthetized by the invading microorganisms [52]. Serine proteinases then stimulate the activation of the cytokine Spätzle and Toll pathways for the expression of antimicrobial peptides [50]. Most transcriptome studies in Lepidoptera have primarily focused on the identification and expression of immune genes as a response to bacterial and/or fungal infections [53,54]. Here, we found evidence that genes at many functional levels of the immune system (i.e. recognition, signalling and antimicrobial peptides) are responsive to antibiotics *per se*, being mainly downregulated (electronic supplementary material, table S1).

### (i) Recognition

Two PRP receptors namely C-type lectins (five genes) and scavenger receptors (three genes) were found downregulated (electronic supplementary material, table S1). Scavenger receptor genes have been previously reported upregulated in the presence of bacterial and fungal peptides in the silkworm (*Bombyx mori*) and in the diamondback moth (*Plutella xylostella*) [55,56]. In the hornworm (*Manduca sexta*), C-type lectins have been shown to bind bacterial lipopolysaccharide, inducing agglutination of bacteria and yeast, helping haemocytes eliminate infections through phagocytosis [57]. More recently, transcriptome analyses of *Gynaephora qinghaiensis* showed C-type lectins being downregulated in response to parasitism [58]. By contrast, the cabbage looper (*Trichoplusia ni*) showed a strong upregulation C-type lectins when infected by baculovirus AcMNPV [59]. Hence, it is clear that in C-type lectins induction varies according to the invader (i.e. fungi, virus or gram ± bacteria), whereas scavenger receptors have a broader recognition spectrum. Our finding of downregulation of both C-type lectins and scavenger receptors suggests that antibiotics may help contain general infections, and thus PRPs are de-activated by the innate immune system.

### (ii) Signalling

Insects respond to infections via the Spätzle and Toll pathways, which are activated by serine proteases signalling cascades for melanization and antimicrobial peptides [60]. Serine proteases circulate as inactive zymogens in the haemolymph and become sequentially activated upon recognition of microbial polysaccharides by PRPs. Serines are inactivated by serpines after the accomplishment of their defensive functions. The balance between the effectors serines and their modulators serpins ultimately determines the susceptibility or resistance to infection [61]. In this study, serines included in the signal modulation group, like serine protease, serine proteinase, and trypsin-like serine proteinase, were found up- and downregulated. This is in agreement with other lepidopteran studies that have found differential regulation in their expression and in their serpin modulators in response to fungi [56], bacteria [62] or parasites [63]. Here, however, serines were mostly downregulated as the number of antibiotic injections increased, whereas serpins were found persistently downregulated (electronic supplementary material, table S1). This suggests that antibiotics may disrupt the immunologic balance by effectively suppressing serine regulators. This could have important consequences as an immunologic unbalance could cause complete immunosuppression, or an over-response with the consequence of self-tissue damage and/or the elimination of beneficial or commensal microbes.

### (iii) Antimicrobial peptides

In Lepidoptera, the most commonly reported peptides against various microbial infections are attacins, cecropins, lebocins, gloverins, gallerimycins, hemolyn and defensins [58,64]. In this study, no known antimicrobial peptides could be detected to have been induced. This is to be expected given the downregulation of recognition and signalling pathways that trigger their synthesis.

Previous studies have tested different immune reactions of the wood tiger moth when challenged with different microbes. Infected larvae of high and low pathogen resistance (i.e. based on cuticular melanin content), with high- and low-virulence strains of *Serratia marcescens*, were reared on diets with and without antimicrobial compounds [65]. The antimicrobial diet enhanced survival only of the high-melanin larvae, which were also more resistant to the low-virulence strain but not the high-virulence strain. In a later study, Mikonranta *et al.* [66] tested the effect of immune priming by feeding pathogenic (*S. marcescens*) or non-pathogenic (*E. coli*) bacteria to wood tiger moth larvae and injected the same bacteria 5 days later. The authors then tested for phenol bactericidal reactive oxygen species (ROS), phenoloxidase (PO) and lytic activity from the haemolymph. Larvae exposed to *S. marcescens* had higher ROS. However, lytic and PO did not differ from the *E. coli* priming. By contrast, [67] reported a high PO activity in larvae that have been fed an antimycotic (fumagillin). [68] simulated ectoparasitism by implanting nylon threads on larvae and measured the encapsulation response (i.e. darkening of the implant), as well as the lytic activity in

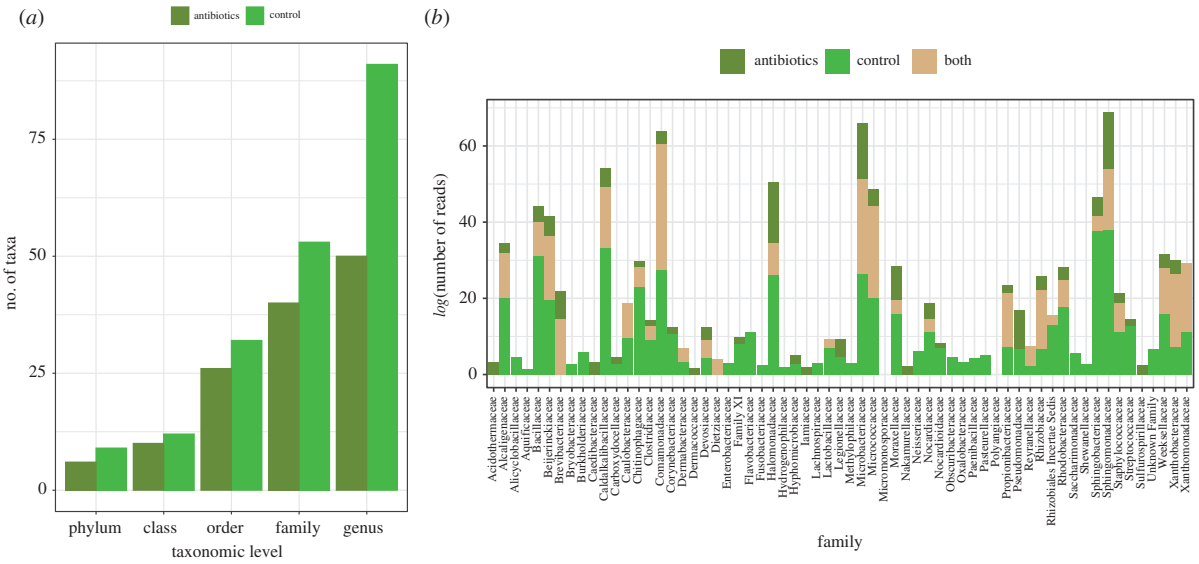


**Figure 3.** (*a*) Number of bacterial taxa per taxonomic level not found in wood tiger moths (*Arctia plantaginis*) treated with antibiotics. (*b*) Bacterial families and their abundance present in samples treated with antibiotics, or in the control group, or in both. (Online version in colour.)

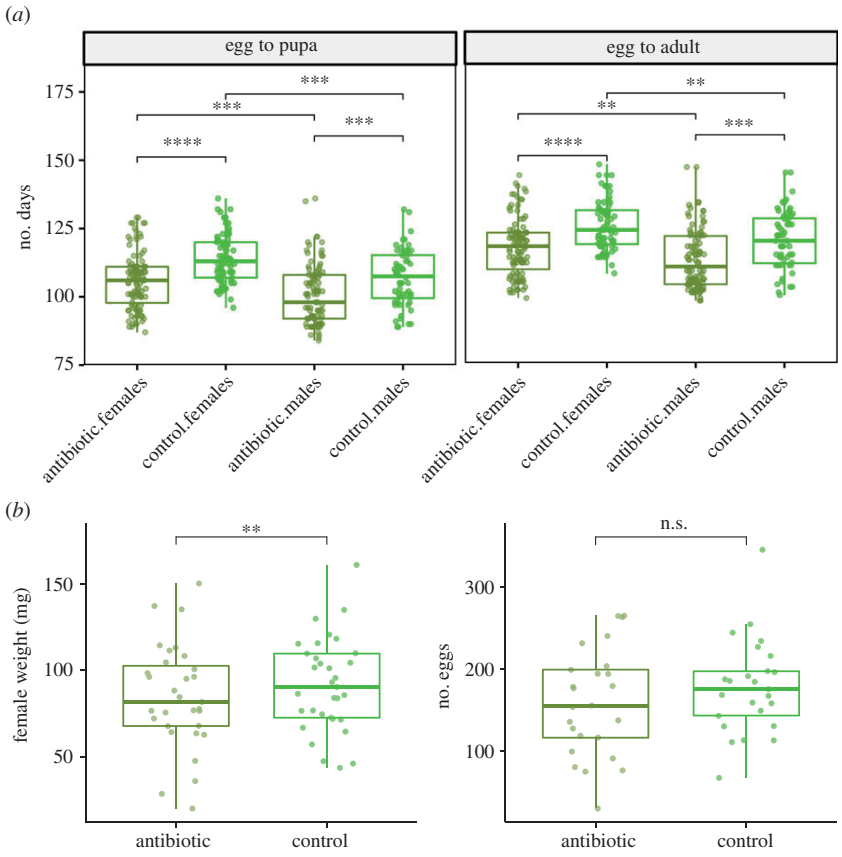


**Figure 4.** (*a*) Life-history traits of the wood tiger moth (*Arctia plantaginis*) under antibiotics and control treatments. The number of days elapsed from egg hatching until pupation and adult stages are shown for both sexes. Boxplot shows the median and the interquartile range to the 25th and 75th percentile. ****: $p <= 0.0001$, ***: $p <= 0.001$, **: $p <= 0.01$. (*b*) Adult female weight and number of eggs produced in the antibiotics and control treatments. (Online version in colour.)

the haemolymph. The results showed that adults of different colours reacted differently in their encapsulation response and lytic activity. Altogether, these previous studies indicate that wood tiger moth can mount immune reactions against different pathogens (i.e. parasites, gram ± bacteria or fungi), and the effectiveness of such immune reactions greatly depends on host condition, colour morph, diet and pathogen virulence. In the present study, we add to this existing

knowledge by showing that the genes involved in recognizing, signalling and mounting immune responses can be suppressed in the presence of antibiotics.

### (c) Bacterial load

Microorganisms are ubiquitous in insects. It is estimated that roughly 70% of species host one or more microorganismal symbionts [69]. Microorganisms can profoundly impact insects' physiology, ecology and evolution [70]. Bacterial lineages, in particular, have evolved diverse mechanisms to gain entry and proliferate in the tissues and cells of insect hosts [71]. Bacteria can have a substantial influence on growth and immune processes. For instance, gut bacteria have a major role in providing essential nutrients for their insect host [72], whereas the hosts' immune system promotes the growth of beneficial bacteria and helps maintain a stable microbial community [73]. Hence, by perturbing the bacterial load, the cross-talk between immunity and growth can be impacted. Here, antibiotics significantly reduced bacterial abundance and diversity (figures 2 and 3). Some of the bacteria removed or depleted are known to be pathogenic for insects (i.e. entomopathogenic). Notably, bacteria from *Bacillus* and *Acinetobacter* genera are highly toxic to some Lepidoptera species [74–76] (electronic supplementary material, table S6). It can be envisaged that the depletion of pathogenic bacteria by the antibiotics freed resources allocated to keep the bacteria at bay, which could then be reallocated to growth. This is supported by the downregulation of immune genes and the upregulation of growth-related genes as a response to antibiotics. However, it is unclear if the moth's relaxed immune activity and growth increase are due to a depletion of toxigenic bacteria, or simply to a reduction in the bacterial load. An increased bacterial diversity may require higher immune responses to constrict abundances of multiple taxa that could upset homeostasis if left to multiply unchecked. These taxa would not have to be pathogenic, it could merely upset chemical balances in the microbiome and subsequent potential functions it provides to its host. Further studies using targeted antibiotics are needed to discriminate the host's immune reaction to toxins and to symbiotic bacteria. In any event, our results indicate that a disrupted microbiota can have a significant impact in the interaction between growth and immunity.

### (d) Life histories

We observed faster growth and higher survival rates of antibiotic-treated larvae, which is in agreement with previous findings in several taxa [3,77–79]. This can be advantageous for today's insect mass-rearing for different purposes like pest control, commerce and research. Aside from the advantages for the husbandry side, plasticity in growth rate should provide some advantage to the moth itself, otherwise why invest in faster growth when the chance arises? In non-seasonal environments, for instance, a faster growth rate can be seen as a major advantage for insects as the potential to die before reproduction is reduced [80].

While the control larvae took longer to develop, once they reached adulthood, the emerging females were significantly heavier than the antibiotic-treated group (figure 4*b*). This is congruent with previous findings where antibiotics were fed to pre-diapausing larvae of the wood tiger moth [67]. It is commonly accepted that heavier weight translates into greater fecundity [81], as was shown by Dickel *et al*. [67], where heavier control females laid more eggs than females that had been feed with antibiotics. In the present study, however, we found no differences in the number of eggs even though control females showed a heavier weight. This suggests compensatory mechanisms presumably operating during the pupal stage in which the effect of antibiotics was not observed. Alternatively, the contrasting results could be due to different sampling strategies. In [67], the number of laid eggs was counted, whereas in this study, we counted the number of eggs inside the females. In addition, here, we use a combination of two broad-spectrum antibiotics injected into the larvae, whereas in [67], a fungicide was fed to the larvae. At the moment, it is unclear if wood tiger moth females lay all the eggs they produce. Future studies should consider the number of hatched larvae as a metric to evaluate the effect of antibiotics in both, male and female fecundity.

## 5. Conclusion

We found evidence that by perturbing the microbial community with antibiotics, resource allocation trade-offs can be generated between high-resource-demanding processes such as growth and immunity. Our main finding of downregulation of the immune system could have important implications for several taxa. For instance, while there are marked differences between insect and mammal immune systems, there are also many conserved similarities in their innate immunity due to a common evolutionary origin [82]. Both systems consist of humoral and cellular responses involving processes such as recognition, signalling cascades and antimicrobial peptide secretion. Several insect taxa (i.e. *Drosophila melanogaster, Galleria mellonella, Manduca sexta* and *Bombyx mori)* are increasingly being used in the medical field to overcome the disadvantages associated with testing in mammalian systems (e.g. cost, housing and legal/ethical restrictions) while generating comparable results [83]. Prophylactic antibiotic treatment is a common practice in the medical field. However, the interacting effects of antibiotics with other fundamental processes, such as modulation of the immune system, are surprisingly understudied at the molecular level. Thus, the paradigms set in insects can serve to guide disease development (i.e. transmission and virulence) of medically important pathogens. This is also true for commercial livestock, for which there is growing evidence of antibiotic resistance and its transmission to humans due to antibiotic administration [2]. Finally, for insect farming, either for research or conservation-management purposes, the results obtained here can inform producers about the potential negative side effects of antibiotics as growth promoters, such as immune suppression.

Data accessibility. All sequence data have been deposited in the National Center for Biotiechnology and Information (NCBI) under Bioproject PRJNA557336. The code to generate statistics and figures is given in the electronic supplementary material [84].

Authors' contributions. J.A.G.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing-original draft, writing-review and editing; L.M.: data curation, formal analysis, investigation, methodology, writing-review and editing; J.M.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing-review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. The authors declare no competing interests.

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