Host-microbiome-pathogen interactions in cockroaches

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Hereby I confirm that I have prepared my doctoral thesis independently and without impermissible help.

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List of abbreviations

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
Antimicrobial peptide	AMP
Balanced	B
Berlin Center for Genomics in Biodiversity Research	BeGenDiv
Carbohydrate	C
Colony-forming units	CFUs
Conventional type	C-type
Dual oxidase	DUOX
Enterobacter sp.	*Esp_Z*
False discovery rate	FDR
Federal Institute for Materials Research and Testing	BAM
Generalized linear mixed model	GLMM
Gram-negative binding protein	GNBP
Lipopolysaccharide	lps
Liquid chromatography-mass spectrometry	LC-MS
Lysogeny-broth	LB
Lysyl endopeptidase	LysC
Macrophage mannose receptor	MMR
Mass spectrometry	MS
Niemann-Pick disease type C intracellular cholesterol transporter 2	NPC2
Optical density at 600 nm	OD_{600}
Pattern recognition receptor	PRR
Peptidoglycan recognition protein	PGRP
Post-infection	p.i.
Prophenoloxidase	proPO
Protein	P
Reactive oxygen species	ROS
RNA-mediated interference	RNAi
RNA-sequencing	RNA-seq

General introduction

A new concept of the term ‘self’ – the host, the microbiome and the pathogens

A classical philosophical approach to describe individuality is the discrimination of self and non-self. These terms strongly influenced nowadays vocabulary of immunology (Pradeu 2019). Self, in this case, means biological individuality featuring four different main elements: cohesion, delineation, uniqueness and persistence (Pradeu 2016; Santelices 1999). A common belief in the field of biology is that immunity is a key factor of shaping individuality since it is building the borders to the non-self environment (Pradeu 2012). Everything which is self, so part of the host, should therefore be accepted and everything which is non-self, especially harmful pathogens, should be rejected by the immune system (Burnet 1969).

To discriminate self and non-self, the immune system of vertebrates features innate and adaptive immunity whereas invertebrates only feature innate immunity. Innate immunity is a non-specific first-line defence against microbes (Iriti and Faoro 2007; Abbas *et at.* 2012). It consists of cellular and humoral defence mechanisms which are in place even before the outbreak of an infection (Abbas *et al.* 2011). In contrast, adaptive immunity is a very specific response to an infection. It adjusts to it and increases in its defensive power with each re-exposure to a particular microorganism. Its hallmark features are exquisite specificity and the ability to remember repeated exposures with the same pathogen (Abbas *et al.* 2011).

In reality, the discrimination of self and non-self is not as simple as that. In the case of autoimmune diseases, the immune system of an individual attacks its own healthy cells and tissues or the immune system gets rid of dead cells (phagocytosis) or repairs tissue (Rankin and Artis 2018). Another fact to consider is the individuals own microbiota, called the microbiome, which is tolerated by the immune system because of its various beneficial functions explained later (Chu and Mazmanian 2013). Therefore, we should switch from a very internalist point of view to a more tripartite interactionist point of view, because the interactions between the host, the pathogens and the microbiome in the environment in the end lead to and influence what self, so individuality, is (Fig. 1.1; Pradeu 2012; Brinker *et al.* 2019). As a result, one should consider hosts no longer as autonomous entities but as complex networks or ecosystems, in which not only the microbiome interacts with the host forming the ‘holobiont’, but also in which the microbiome interacts with other microbes and other hosts (McFall-Ngai *et al.* 2013; Bordenstein and Theis 2015; Brinker *et al.* 2019).

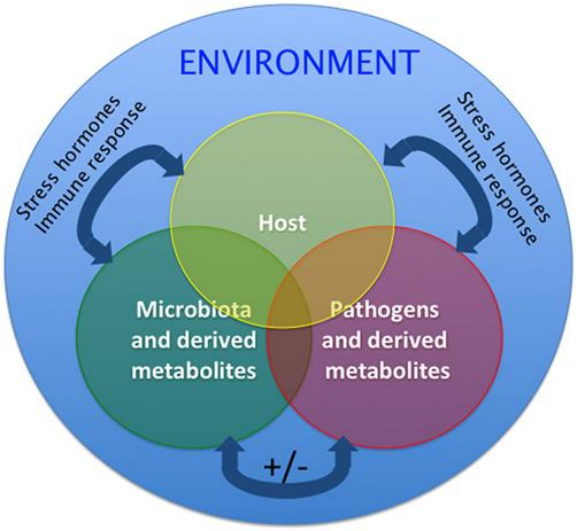

Fig. 1.1: Tripartite interactions between host, microbiome and pathogens in the environment (taken from Kelly and Salinas 2017).

The role of the microbiome

The microbiome, as termed by Joshua Lederberg, is the total ecological diversity of microorganisms which reside certain habitats or ecosystems (Saleem 2015; Lederberg and McCray 2001). It is the major component of global biodiversity occupying central roles in ecosystem functions including nutrient cycling, host fitness and virulence (Saleem 2015). Therefore, the microbiome can fulfil many different functions in the tripartite structure (host, pathogens, microbiome). Its symbiotic relationships to the host can be parasitic (pathogenic), commensal or mutualistic (Barton and Northup 2011). In parasitism the host is harmed while the parasite benefits; in commensalism the host is not affected while the commensal benefits; and in mutualism both the host and the mutualist benefit (Barton and Northup 2011). Thus, those relationships are strongly influencing the expression of the self (Moya *et al.* 2008). Microbiomes are associated with all kinds of eukaryotic taxa (Fig. 1.2; Saleem 2015) and shaped by diet, body size (Reese and Dunn 2018) and the host's immune system (Mistry *et al.* 2017; Thaiss *et al.* 2016). Diet can shape, for example, the gut microbial community directly through the ingestion of microorganisms in food and favouring of taxa which can best utilize food-derived nutrients or indirectly through the impact of food on gut anatomy, digestive function and/or immunity (reviewed in Douglas 2015).

Microbes can also be transferred vertically via reproductive propagules between individual hosts and/or horizontally via a free-living phase between host-associated and free-living microbial communities (Dittmer *et al.* 2016; Adair and Douglas 2017).

Additionally, microbiomes act as extended genomes of their hosts, called ‘hologenomes’ (Zilber-Rosenberg and Rosenberg 2008). Consequently, microbiomes are extremely diverse in their taxonomic, genomic, physiological and morphological composition (Fig. 1.2; Saleem 2015).

Obligate symbionts, which are necessary for the survival and fertility of their hosts (Louie 2013), are inherited by strict vertical transmission and they are often housed in specialized host cells, the bacteriocytes (Moya *et al.* 2008). For most parts, they have nutritional functions providing the host with nutrients that are deficient in its diet (e.g. essential amino acids, vitamins and cofactors). In return, the microbes gain permanent supply with a variety of metabolites (reviewed in Moya *et al.* 2008).

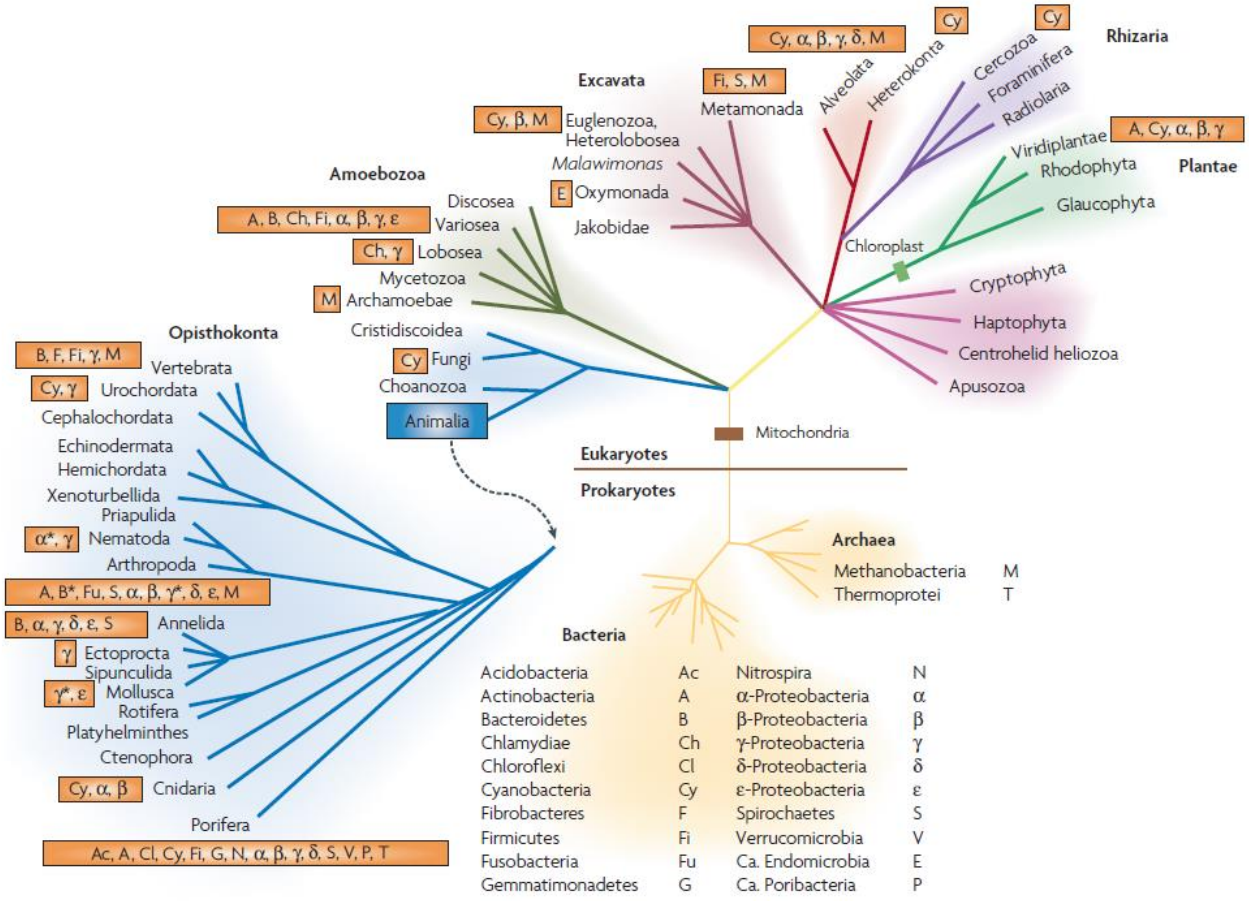

Fig. 1.2: The phylogenetic distribution of symbioses, indicating the bacterial and archaeal classes within which there are associations with eukaryotic hosts (taken from Moya *et al.* 2008).

Facultative symbionts can be either beneficial but not essential for the survival and/or fertility of their hosts (Louie 2013) or even deleterious for them (Dale and Moran 2006). They can be transmitted vertically or horizontally, and they can reside multiple host tissues and cells (Baumann 2005; Russell *et al.* 2003). Without considering pathogens facultative endosymbionts maintain themselves in host populations either via mutualism or through reproductive manipulation (Minelli *et al.* 2013). Tasks of beneficial microbes can include saviour from heat damage, protection against parasites and pathogens or a participation in host specialization on, for example, exclusive plants (reviewed in Moya *et al.* 2008).

The microbiome of insects

Insects are the most successful animal class on Earth with approximately 5.5 million different species which can survive in various ecological niches (Stork 2018; Krishnan *et al.* 2014). Their associated microbiomes are equally if not more diverse, including bacteria, archaea, fungi, protozoa and viruses (Engel and Moran 2013). The evolutionary success of insects depends at least in part on their relationships with these microorganisms (Engel and Moran 2013). Most of the cells in an insect are of microbial origin and the insect's microbiota makes up for 1-10 % of the insect's biomass (Douglas 2015). The primary habitats for these microbes are located in all three regions of the gut, namely the foregut, the midgut and the hindgut (Fig. 1.3a; reviewed in Kaufman *et al.* 2000). Other sections of the insect which are inhabited by microorganisms are the easily accessible cuticle and microorganisms which can breach this exoskeleton or the walls of the gut can also colonize the hemocoel or other insect cells like bacteriocytes or mycetocytes (Douglas 2015). Additionally, insects can promote dominant microbial taxa due to behavioural actions including coprophagy (the eating of faeces), trophallaxis (transfer of gut fluids by anus-to-mouth or mouth-to-mouth feeding) and maternal smearing of gut microorganisms on the eggshell, which is taken up by the descendants during hatching (Beaver 2010; reviewed in Douglas 2015).

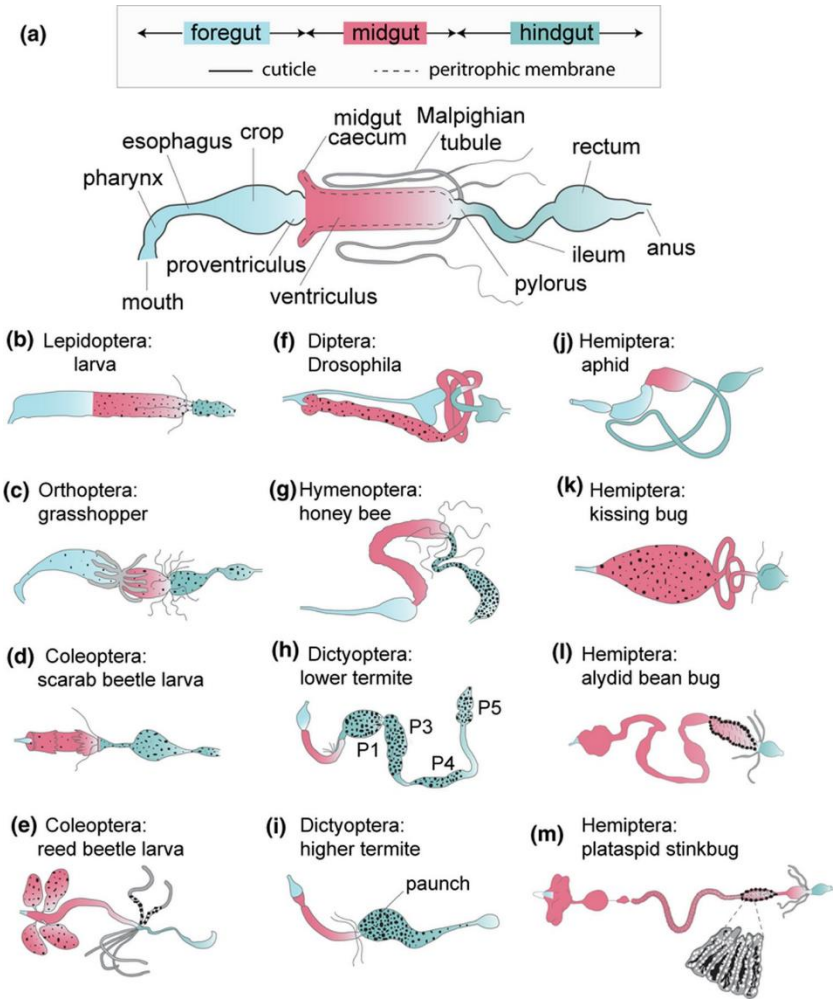

Fig. 1.3: (a) Generalized gut structure of insects. (b) – (m) gut structures from insects of different orders. Light blue indicates the foregut, magenta the midgut and grey-blue the hindgut. Present microbiota is indicated by black dots (taken from Engel and Moran 2013).

Common bacterial phyla in all kinds of insects are Proteobacteria (Gamma-proteobacteria, Alphaproteobacteria, Betaproteobacteria), Bacteroidetes, Firmicutes, Clostridia, Actinomycetes, Spirochetes, Verrucomicrobia and Actinobacteria, while in some rare cases other phyla dominate certain specialized insect groups (reviewed in Engel and Moran 2013; reviewed in Douglas 2015). For instance, Spirochaetae, Fibrobacteres, and the candidate phylum TG3, are exclusively found in wood-feeding termites (Köhler *et al.* 2012; Warnecke *et al.* 2007). Methanogenic and non-methanogenic archaea of the phylum Euryarchaeota, are usually found in the hindguts of beetles, cockroaches, termites, and millipedes (reviewed in Gurung *et al.* 2019). Fungi, which colonize insects are mostly ascomycetes belonging to the Clavicipitaceae and the Saccharomycetes (Douglas 2015). Finally, protozoa that colonize insects include protists (Metamonada) and anaerobic ciliates

(Clevelandellida) in cockroaches and termites as well as Trypanosomatida in hemipterans, hymenopterans and dipterans (reviewed in Douglas 2015). Concerning viruses, insects can not only be vectors (e.g. for Ebola or Zika) they can also be infected with them. This sometimes leads to drastic behavioural changes *e.g.* increased egg laying (Gandon *et al.* 2009) or change of migration behaviour in the case of the famous baculovirus (van Houte *et al.* 2014), which makes viruses potent routes for biological pest control (Gurung *et al.* 2019). Additionally, viruses could also benefit their host and they can even infect the other members of the insect microbiome (Gurung *et al.* 2019).

Overall, the diversity of the microbiome greatly benefits their insect hosts by providing nutrients, protection against parasites and pathogens or detoxification of plant chemicals and insecticides.

Nutritional role of the insect microbiota

One important factor why insects have become one of the most successful lifeforms on earth is because they are adapted to a vast range of ecological niches, where they often feed on nutrient-poor or refractory diets and therefore compensatory nutritional symbioses are widespread (Bourtzis and Miller 2003; Engel and Moran 2013).

In most cases microbes provide their hosts with special nutrients that are absent in the food and cannot be synthesized by the host itself (Dillon and Dillon 2004). These include essential amino acids and nitrogen as well as B vitamins and sterols (Dillon and Dillon 2004; Douglas 2017) In addition, as insects consume complex foods like plant leaves, microbes assist in the degradation of complex polysaccharides of plant fibres like cellulose and in the digestion of food or its efficiency in general (Dillon and Dillon 2004; Douglas 2015).

The xylem and the phloem of plants have unbalanced amino acid profiles containing less than 20 % of essential amino acids (Douglas 2006; Douglas 2009; reviewed in Hansen and Moran 2014). Sap feeding insects overcome these limitations with the help of their microbial symbionts (reviewed in Hansen and Moran 2014). Aphids (Fig. 1.3j), for example, harbour intracellular bacteria of the genus *Buchnera* which provide essential amino acids (reviewed in Douglas 1998) and long horned beetles (*Anoplophora glabripennis*) derive essential amino acids from their symbiotic gut microbiota as well (Ayayee *et al.* 2016). Additionally, some microbes simply concentrate nitrogen for their insect hosts. For instance, some termite species cultivate a fungus which has nitrogen-rich nodules on which the termites feed (Douglas 2009). Interestingly, cockroaches, the nearest relatives of termites, also harbour endosymbionts

(*Blattabacterium sp.*) capable of ammonia recycling and essential amino acid synthesis (Sabree *et al.* 2012).

In the case of some phytophagous insects like the African cotton stainer (*Dysdercus fasciatus*; Salem *et al.* 2014), aphids (Shigenobu *et al.* 2000) or beetles (Cerambycidae and Anobiidae; Douglas 1989) and blood-feeding insects like bed bugs (*Rhodnius prolixus*; Eichler and Schaub 2002), tsetse flies and lice (Douglas 2011) B vitamins are also provided by the microbiota.

Furthermore, phytophagous insects, especially the ones feeding on wood, often harbour microbes participating in degradation of cellulose into simple sugars in their gut (reviewed in Engel and Moran 2013). In addition, plant cells are protected by lignin and therefore many insects can only feed on predegraded wood, but in *A. glabripennis* fungi and bacteria break it down (reviewed in Geib *et al.* 2008 and Engel and Moran 2013). Nevertheless, the best studied nutritional gut mutualists when it comes to degradation of plant materials are those found in termites (Engel and Moran 2013).

The termite's symbiotic gut microbiota plays a crucial role in the digestion of lignocellulose, which is the most abundant biomass on earth and mainly comprised of cellulose, hemicelluloses and lignin (Brune and Ohkuma 2010; reviewed in Ni and Tokuda 2013). Furthermore, termites are categorized based on the presence of certain members of the microbiota, the flagellated protists, into lower (protists present) and higher termites (protists absent; Ni and Tokuda 2013). In lower termites (Fig. 1.3h; families: Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Serritermitidae) and the closely related wood-feeding cockroaches of the genus *Cryptocercus* lignocellulose is mostly broken down by protists in the hindgut (reviewed in Brune and Ohkuma 2010 and in Ni and Tokuda 2013). Hydrogen which results from this breakdown is rapidly removed via bacteria and archaea through reductive acetogenesis and methanogenesis (reviewed in Engel and Moran 2013). In contrast, higher termites (Fig. 1.3i; family: Termitidae) which represent more than 80 % of all termite species today evolutionary lost protists at some point in the Eocene (Brune 2014). Therefore, lignocellulose degradation is done by the cellulolytic activity of bacteria in the hindgut (Warnecke *et al.* 2007; Köhler *et al.* 2012) which are also involved in acetogenesis and nitrogen fixation (Warnecke *et al.* 2007; Burnum *et al.* 2011).

Lastly, in house crickets (*Acheta domesticus*), the utilization of soluble plant polysaccharides is increased by bacteria in their hindgut (reviewed in Dillon and Dillon 2004) and in cockroaches the gut microbiota appears to be involved in the degradation of plant polymers as well (Hackstein and Stumm 1994; Zurek and Keddie 1998).

Microbial impacts on insect detoxification

Many secondary plant metabolites are defensive compounds that are either toxic to insects or deter feeding (Douglas 2013). In herbivorous insects the microbiome acts therefore as 'microbial brokers' by helping their hosts to detoxify allelochemicals like glucosides, flavonoids, tannins, and alkaloids (reviewed in Dillon and Dillon 2004; Engel and Moran 2013). This is of particular importance since certain plants are only available as food sources if the toxins can be neutralized (Engel and Moran 2013).

Cigarette beetles (*Lasioderma serricorne*), for example, become susceptible to the tannins in their host plants when their symbiotic yeast (*Symbiotaphrina kochii*) was experimentally removed through antibiotic treatment (reviewed in Itoh *et al.* 2018). Additionally, in *Tenebrio molitor* larvae the gut microbiota can hydrolyse toxic glucosides which could help the beetle to adapt to local food types (Genta *et al.* 2006) and the fungus (*Leucoagaricus*) of attine ants is a form of pre-gastric detoxification of plant secondary compounds (reviewed in Douglas 2009).

Furthermore, recent studies could show that the symbiotic microbiota can help to overcome pesticides used in the control of insect pests as well (reviewed in Itoh *et al.* 2018). Stinkbugs (*Riptortus pedestris*; Fig. 1.3m) can environmentally acquire a bacterium (*Burkholderia*) which hydrolyses the insecticide fenitrothion in the insect's gut (Kikuchi *et al.* 2012).

There is also evidence from studying termites, cockroaches and hemipterans that the microbiome can recycle nitrogenous waste products. In the case of termites, those microbes are *Bacteroides* and *Citrobacter* bacteria species, in the case of cockroaches it is the *Blattabacterium sp.* bacterium and in the case of hemipterans it is in most cases the fungi *Nilaparvata lugens* (reviewed in Douglas 2009).

Microbial impacts on insect parasite and pathogen protection

It has been shown that variation in resistance and immunity against pathogens and parasites is associated with the presence or absence of symbionts in different insect species (Feldhaar 2011). Colonization with commensal or mutualistic microbiota can already increase the resistance of hosts against invasion by pathogens or parasites due to nutrient competition, niche occupation or even immune priming (reviewed in Engel and Moran 2013). Another indirect way how symbionts might confer protection is through priming/stimulating the host immune system. There are several independent studies on *Anopheles* mosquitoes showing that the gut microbiota especially the bacterium *Enterobacter sp.* (*Esp_Z*) protects them

against *Plasmodium* infections (reviewed in Engel and Moran 2013; Cirimotich *et al.* 2011). Based on RNA-sequencing (RNA-seq) studies it was assumed that the protection was the result of microbiota induced immune responses resulting in the upregulation of host antiplasmodial factors (reviewed in Engel and Moran 2013). Thereby, it may be possible to manipulate the composition of the midgut microbial flora in wild mosquitoes to increase the prevalence of *Esp_Z* or other naturally inhibitory bacteria to combat the spread of malaria (Cirimotich *et al.* 2011). Moreover, there is evidence that the microbiome can directly attack parasites and pathogens and most of these beneficial microbes are heritable, which means that they are reliably transmitted from parent to offspring (Oliver *et al.* 2014). Protection against parasitiods may be a widespread phenotype conferred by heritable bacteria given the number, specificity and intimacy of these interactions (Oliver *et al.* 2014). For instance, in aphids the bacteria *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica*, contribute to protection against parasitic wasps (reviewed in Feldhaar 2011). In the case of *H. defensa* it has been shown that it, together with an associated phage, actively kills wasp larvae by producing toxins (reviewed in Brownlie and Johnson 2009; reviewed in Feldhaar 2011). Another example involves *Drosophila hydei* which is protected by a heritable *Spiroplasma* symbiont against parasitism by the wasp *Leptopolina heterotoma* (Xie *et al.* 2010). Entomopathogenic nematodes are another important group of natural enemies of insects, but to date, only one example of symbiont-based defence exists (Oliver *et al.* 2014). In this case the *Spiroplasma* symbiont is again involved by protecting *D. neotestacea* against *Howardula aoronymphium* (Jaenike *et al.* 2010). Furthermore, the microbiota of insects can also protect against infections with entomopathogenic fungi. This is, for example, the case for the already mentioned *R. insecticola* protecting aphids from the fungus *Pandora neoaphidis* (Scarborough *et al.* 2005). Protection against pathogenic fungi is also conferred in digger wasps by a *Streptomycete* bacterium harboured in antennal segments of females (Kaltenpoth *et al.* 2005). Additionally, fungus-growing beetles are protected in a similar manner by *Streptomycete* bacteria (Scott *et al.* 2008) as well as attine ants which are protected by *Pseudonocardia* and *Amycolatopsis* bacteria (Sen *et al.* 2009).

There is also evidence that intracellular *Wolbachia* bacteria which are maternally inherited and found in at least 20 % of all insect species (reviewed in Brownlie and Johnson 2009) enhance survival against and/or reduces load of RNA viruses in *Drosophila* (Glaser and Meola 2010; Teixeira *et al.* 2008; Hedges *et al.* 2008), mosquitos (*Culex quinquefasciatus* and *Aedes aegypti*; Glaser and Meola 2010; Moreira *et al.* 2009).

Finally, the insect microbiota can combat bacterial pathogens as well. Honeybees for instance harbour lactic acid bacteria in their crop which are effective against a broad range of bacteria

that are present in nectar and pollen (Vásquez *et al.* 2012). Thereby, the colony is protected from pathogens introduced by foraging workers.

Nutritional impacts on insect pathogen protection

Not only the microbiome but also the nutrition of the insect host can strongly influence the outcome of an infection (Lazzaro and Little 2008; Schmid-Hempel 2011). This makes an already complex relationship even more complicated, since all the members of the microbiome receive food virtually from the host (Fig. 1.4; Ponton *et al.* 2013). However, they all differ in their own needs and their contribution to host fitness (reviewed in Ponton *et al.* 2013).

When it comes to parasites and pathogens the host can be considered simply as a growth medium since pathogens are either hijacking the host's food or they are directly feeding on the host's tissues and fluids (Ponton *et al.* 2013). Therefore, a sick individual might be forced to drastically alter its feeding behaviour upon infection to adjust its nutrient intake to compensate the resource competition with parasites and to accommodate the demands of fighting the infection (reviewed in Ponton *et al.* 2013). Historically, the impact of nutrition on host immune functions was investigated by examining the influences of caloric restriction or starvation (reviewed in Chambers and Schneider 2012) but nowadays it becomes apparent that the macronutrients protein (P) and carbohydrate (C) seem to be of special importance as shown by several independent studies. For example, Graham *et al.* (2014) could show, that in Australian plague locusts (*Chortoicetes terminifera*) increased consumption of C protects them against a fungal infection with the biopesticide *Metarhizium acridum*. Moreover, dietary P quality influences melanisation and immune function (phenoloxidase (PO) activity and lysozyme-like antibacterial activity) in the caterpillar *Spodoptera littoralis* (Lee *et al.* 2008a). Additionally, caterpillars infected with a generalist gram-positive bacterium (*Bacillus subtilis*; Povey *et al.* 2009) or a DNA virus also prefer P over C (Lee *et al.* 2006).

However, as briefly mentioned before, reducing the overall food intake can impact the outcome of an infection, a phenomenon called illness induced anorexia which is well-documented in insects (Adamo *et al.* 2007; Ayres and Schneider 2009). One theory is that thereby hosts can limit the nutritional resources available to the pathogen (Kluger and Rothenburg 1979).

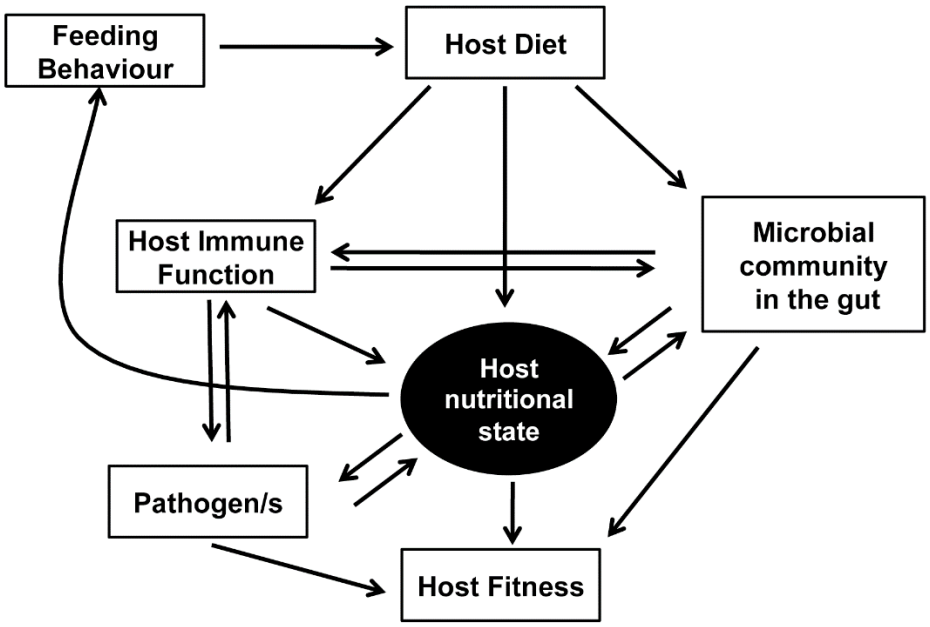

Fig. 1.4: The interaction network of insect hosts, their gut microbiota and pathogens (Ponton *et al.* 2011a).

Cockroaches as a great model to study the tripartite of hosts, their microbiomes and pathogens

Cockroaches are found all across the globe with around 4,600 valid species described and they are popularly considered to be one of the oldest terrestrial arthropod groups because of fossil records which date back to 350 million years ago and their simple body plan (Beccaloni 2014; Bell *et al.* 2007; Cochran 2009). Thirty species are also renowned for being pest species that infest human buildings and threaten public health by transmitting diseases (Cochran 2009). Additionally, they represent model organisms in research, particularly in the fields of reproductive physiology, neurobiology, behavioural biology (Costa 2006), social evolution and applied medicine as a source of novel antimicrobial agents (reviewed in He 2018).

Beside this, cockroaches are also reasonable models to study the complexity of the host-microbiome-pathogen tripartite. The tripartite is often only investigated in its parts with several studies either focusing only on the hosts or only on the microbiome perspective when it comes to pathogen infections. Cockroaches are highly suitable for studies bridging this gap, especially if nutrition is also a concern, for several reasons.

Firstly, they are omnivorous generalists (Bell *et al.* 2007; Cochran 2009) which makes manipulating their food very easy since they will basically feed on all kinds diets if they need to.

Secondly, they harbour a diverse microbiota. The American cockroach *Periplaneta americana*, for example, harbours hundreds of microbial species. Its core gut microbiome is composed of bacteria of the Bacteroidetes and Firmicutes phyla while there are also members of the Euryarchaeota, Actinobacteria, Proteobacteria, Synergistetes, Tenericutes, and Verrucomicrobia phyla as well as multiple unclassified bacteria present (Tinker and Ottesen 2016). In contrast, in *Blattela germanica* the dominant phyla of the core gut microbiome are Bacteroidetes, Firmicutes, Proteobacteria and Fusobacteria (Pérez-Cobas *et al.* 2015).

Thirdly, there are techniques available either by surface sterilization of ootheca (egg pockets) or by antibiotic treatment of the hatchlings to raise, for example, individuals of *B. germanica* (Benschoter and Wrenn 1972; Doll *et al.* 1963; Rosas *et al.* 2018; Zhang *et al.* 2018) and *Shelfordella lateralis* (Tegtmeier *et al.* 2016) in the absence of their conventional microbiome (germ-free). These tools make it possible to identify the role of the microbiome on host physiology and immunity.

Fourthly, they feature effective strategies to combat pathogens since they are frequently exposed to a rich antigenic environment due to their lifestyle (Mayer *et al.* 2016) which makes them well suited for host-parasite interaction studies. These strategies include behavioural defence mechanisms like avoidance of infected conspecifics, grooming or behavioural fever as well as physiological defence mechanisms (reviewed in He 2018). The physiological defence mechanisms consist of both a cellular and a humoral immune response. The cellular response includes phagocytosis and encapsulation (reviewed in He 2018). The humoral response includes the production of several antimicrobial peptides (AMPs; reviewed in He 2018) as well as, similar to other insects, coagulation and melanisation cascades, and the production of reactive intermediates of oxygen and nitrogen (reviewed in Lavine and Strand 2002). Additionally, the recently published genomes of *B. germanica* (Harrison *et al.* 2018) and *P. americana* (Li *et al.* 2018) revealed the expansion of specific immune gene families which further underlines the adaption to antigenic environments.

Description of the project

In this study, I investigated the host-microbiome-pathogen tripartite using the cockroach species *Blatta orientalis* to study the nutritional impacts on pathogen protection and *B. germanica* to study the influence of its microbiome on ontogeny, physiology as well as immunity. The pathogen I used to examine the interactions of the tripartite is *Pseudomonas entomophila* an entomopathogenic, Gram-negative bacterium (Kahlon 2016). It was first isolated from a *D. melanogaster female* and it is a great model to study host-pathogen

interactions since it can effectively infect a broad range of insects including *Drosophila* flies, *Bombyx mori* silk moths and *A. gambiae* mosquitos (Dieppois *et al.* 2015; Kahlon 2016).

In **Chapter I**, I investigated anorexia and macronutrient manipulation upon *P. entomophila* infection in the cockroach *B. orientalis*. I recorded macronutrient preferences to detect shifts in host macronutrient dietary preference and quantity following a sublethal bacterial infection. Additionally, I compared the survival of uninfected individuals on P- or C- enriched diets. I then carried out a quantitative proteomic analysis and an antimicrobial activity assay of hemolymph from *P. entomophila*-infected individuals that had been restricted to diets with defined macronutrient compositions. Furthermore, I followed the survival of *B. orientalis* males restricted to P-rich, C-rich or balanced (B) diet after lethal infection with *P. entomophila*. I showed that diets enriched for P decreased survival of unmanipulated cockroaches. Nevertheless, following immune challenge by *P. entomophila*, cockroaches significantly reduced their overall nutrient intake, particularly of C, and increased the ratio of P (P:C) consumed. It was intriguing that these behavioural shifts did not improve cockroach immunity or survival, with negligible differences in immune protein abundance and antimicrobial activity shown by the proteomic analysis and bacterial growth inhibition assays.

In **Chapter II**, I established a germ-free cockroach system in *B. germanica*. In a first approach this was achieved by surface sterilization of mature ootheca with peracetic acid and sodium hypochlorite. The sterility of adult cockroaches was checked by plating them on lysogeny-broth (LB) and by 16S rDNA sequencing to examine the bacterial community in conventional cockroaches and to account for the presence of unculturable bacteria in germ-free ones. Notably, this resulted in germ-free cockroaches only carrying their vertically transmitted symbiont *Blattabacterium sp.* in 40 % of the cases. However, I was able to improve this to 99 % by feeding the antibiotics rifampicin and gentamicin to freshly hatched nymphs. Exploiting this established system, I investigated the influence of the microbiome on cockroach ontology, to be more precise the developmental time from the day of hatching till the day they turned into adults. I could show that the developmental time of conventional microbiota carrying cockroaches is approximately 35 days shorter than for germ-free ones.

In **Chapter III**, I used the germ-free cockroach system to investigate the role of the microbiome in cockroach immunity. I measured the survival of conventional and germ-free cockroaches upon *P. entomophila* infection and I carried out quantitative transcriptomic analyses on both cockroach types. I was able to show, that germ-free cockroaches were much more susceptible to an infection and that the expression of a variety of putative genes including immune-related genes was affected by the presence of the microbiota.

Chapter I

Eating in a losing cause: anorexia and macronutrient manipulation by a cockroach fails to boost immunity

Abstract

1. Host-pathogen interactions can lead to dramatic changes in host feeding behaviour. One aspect of this includes self-medication, where infected individuals consume substances such as toxins or alter their macronutrient consumption to enhance immune competence. Another widely adopted animal response to infection is illness-induced anorexia, which is thought to assist host immunity directly or by limiting the nutritional resources available to pathogens.
2. Here, we recorded macronutrient preferences of the global pest cockroach, *B. orientalis* to investigate how shifts in host macronutrient dietary preference and quantity of C and P interact with immunity following bacterial infection.
3. We find that *B. orientalis* avoids diets enriched for P under normal conditions, and that high P diets reduce cockroach survival in the long term. However, following bacterial challenge, cockroaches significantly reduced their overall nutrient intake, particularly of C, and increased the ratio of P (P:C) consumed. Surprisingly, these behavioural shifts did not significantly improve cockroach immunity or survival, with negligible differences in immune protein abundance and antimicrobial activity between infected individuals placed on different diets.
4. The lack of a benefit of the host's shift in feeding behaviour highlights a possible decoupling of dietary regulation from immunity in these invasive animals.

Keywords: nutritional ecology, immunity, self-medication, proteome, anorexia

Introduction

Microbe symbioses form a fluctuating but universal backdrop to animal life. However, the evolutionary processes that drive animal hosts and their symbionts, including pathogens, operate at different scales and often in opposing directions (Dawkins and Krebs 1979), with the animal immune system acting as a key interface between host and symbiont ecology (Schmid-Hempel 2003). In addition to the core immune system, behavioural mechanisms have attracted increasing attention for their ability to coordinate host responses to infection (Simpson *et al.* 2015; Wong *et al.* 2015). While behaviour is the primary means by which animals interact with the biotic environment, its importance for a wide range of immune-related functions has only relatively recently come to the fore.

Hosts can respond behaviourally before infection has even taken place. This can include avoidance of pathogen transmission areas (e.g. defecation sites) and deterrence of disease vectors (Hart 2011; Moore 2013). Other prominent examples include activities falling within the category of 'social immunity', which among insects can include pathogen detection alarm behaviours (Rosengaus *et al.* 1999); grooming of conspecific group members (Rosengaus *et al.* 1998; Reber *et al.* 2011); removal (Armitage *et al.* 2016) or even destruction of infected individuals (Yanagawa *et al.* 2011). Such mechanisms are well documented in many social insect lineages, where they contribute significantly to a number of prophylactic mechanisms operating within societies (Schmid-Hempel 1998; Cremer *et al.* 2007). Other prophylactic social behaviours include the collection of secondary antimicrobial compounds to prevent microbial growth in the nest environment (Castella *et al.* 2008; Simone *et al.* 2009), in addition to the direct use – typically via feeding – of antimicrobials in both individual and transgenerational prophylaxis (Lefèvre *et al.* 2010; Lefèvre *et al.* 2012; Milan *et al.* 2012; de Roode *et al.* 2013; Kacsoh *et al.* 2013).

Once infection has occurred, the first and principal line of defence is the immune system. Here, behavioural defensive adaptations can also play an important role in regulating or augmenting the response to infection. As with prophylaxis, the role of feeding behaviour has increasingly been viewed as a key mechanism by which animals can respond to infection (Abbott 2014). Here, the selection of novel antimicrobial compounds, or the enrichment of specific dietary elements can be employed as therapeutic treatment against pathogens (de Roode *et al.* 2013). Fruit flies use ethanol therapeutically as well as prophylactically to combat parasitoid wasp infection (Milan *et al.* 2012) whereas parasitoid fly-infected *Grammia* caterpillars mix pyrrolizidine alkaloid-producing toxic plants into the normal diet to assist parasitoid clearance, which comes at the expense of body growth (Singer *et al.* 2004; Smilanich *et al.* 2011).

Infection-induced adaptive changes to feeding behaviour can also involve modifications to the quantity and composition of macronutrients in the diet. Anorexia is a well-documented response to infection in both vertebrates (Johnson *et al.* 1993; Konsman *et al.* 2002) and invertebrates (Adamo *et al.* 2007; Ayres and Schneider 2009) and is thought to assist hosts in limiting nutritional resources available to pathogens (Kluger and Rothenburg 1979). Anorexia may also help by activating components of the immune system that are enhanced under conditions of nutritional stress, such as autophagy (van Niekerk *et al.* 2016a; van Niekerk *et al.* 2016b). In recent years, the balance of macronutrients itself has been examined as a way for animals to regulate the response to infection. In particular, the proportion of P has been shown to be an important criterion in animal choice of diet following infection. In *Spodoptera* moths, larvae select a diet enriched in P following infection with a generalist Gram-positive bacterium and a host-specific DNA virus (Lee *et al.* 2006; Povey *et al.* 2009; Povey *et al.* 2013), leading to enhanced antimicrobial activity in both cases. By contrast, diets enriched in C were selected when *Tenebrio* beetles and *Grammia* caterpillars were infected with a rat tapeworm (Ponton *et al.* 2011b) and a parasitoid fly (Mason *et al.* 2014), respectively. In the latter study, this behaviour was also associated with an enhanced melanisation response.

The use of macronutrients by hosts to regulate immunity could in principle apply to any animal that is not an obligate food specialist. However, virtually nothing is known about the relationship between macronutrient diet choice and immunity outside of a handful of holometabolous, mostly herbivorous, insect larvae. Holometabolous insects undergo complete metamorphosis consisting of distinct larval, a pupal and an adult winged phase, which are typically correlated with vastly different ecologies and corresponding physiological, morphological and immunological conditions (McMahon & Hayward 2016). By contrast, hemimetabolous insects undergo progressive molts where each larval instar closely resembles the adult (Sehnal *et al.* 1996). Our understanding of the interface between diet, behaviour and immunity would greatly benefit from studying animals that vary widely in their taxonomy, ecology and development.

Our study addresses this by examining the interaction between macronutrient feeding behaviour and immunity in the omnivorous cockroach, *B. orientalis*. We investigate the macronutrient preferences of individuals in response to a range of sublethal immune challenges, before examining the impact of macronutrients on host survival, immune competence and finally, the expression of the host's proteome, which captures a critical component of the host's response to a pathogen. Overall, our findings suggest that diet is decoupled from immune regulation in cockroaches.

Materials and Methods

Insects and bacteria

A breeding culture of sequential *B. orientalis* cohorts was established at the Federal Institute for Materials Research and Testing (BAM) in June 2015, initially obtained from the collection at the Federal Environment Agency, Berlin, which consists of a mixed population of 4 independent genetic backgrounds maintained for 50 generations. Each generation consists of a minimum of 150 breeding pairs of cockroaches to minimize the effects of inbreeding. Each experimental cohort was maintained for approximately 190 days in the dark at 26 °C and 50 % humidity. Prior to being placed on experimental (artificial) diets, animals were reared on a mixture of 77.0 % dog biscuit powder, 19.2 % oat flakes and 3.8 % brewer's yeast and supplied with water *ad libitum* and weekly with apple and carrot slices. All experiments were conducted with males to minimise changes in physiology associated with oogenesis. Each individual was used only once in each experiment. For the food choice experiment and the survival on enforced diets, individuals from 3 different cohorts were used. The generalist Gram-negative bacterial pathogen *P. entomophila* (strain L48; DSM No. 28517) which is able to infect a variety of insect orders (Vallet-Gely *et al.* 2010) was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Bacteria were stored at -70 °C until use in experiments.

Artificial diets

The artificial diets used in this study are based on isocaloric diets, as described elsewhere (Lee *et al.* 2006; Povey *et al.* 2013), which were slightly modified to suit cockroach needs. We employed diets containing 35 % C and 7 % P or *vice versa*, or a B diet containing 21 % C and 21 % P. The latter diet was selected for some assays because it resembles the composition preferred by cockroaches infected with a high sublethal dose of *P. entomophila*. The C portion consisted of sucrose while the P portion consisted of casein, peptone and albumin from eggs in a 3:1:1 ratio. Remaining ingredients are listed in Supplementary Tab. 1. Diet blocks of approximately 0.125 cm^3 in size were dried at 50 °C for 2 days before being weighed and given to experimental cockroaches.

Bacterial inoculation

About 200 µl of an overnight culture of *P. entomophila* was mixed in 10 ml fresh liquid medium (according to DSMZ instructions) and incubated at 28 °C and 140 rpm to an optical density at 600 nm (OD_{600}) of 0.55, representing 1.5 x 10^8 CFUs (colony-forming units; determined by plating). The desired concentrations of bacteria were subsequently obtained by diluting bacteria in insect Ringer's solution (0.024 g calcium chloride, 0.021 g potassium chloride, 0.01 g sodium hydrogen carbonate, 0.45 g sodium chloride, 200 ml distilled water). Cockroaches were anaesthetised with CO_2, abdomens swabbed with 70 % ethanol, then injected with 2 µl of bacterial solution directly into the hemocoel using a glass capillary needle inserted between the 3rd and 4th abdominal segment. Sublethal infections (high: 5.8 x 10^5 CFUs, low: 5.8 x 10^3 CFUs) and lethal (4.0 x 10^6 CFUs) doses were determined in pre-experiment injection assays.

Diet choice following sublethal infection

From each of 3 cohorts, 40 *B. orientalis* males (120 in total) were given free choice of macronutrients by placing them together with 1 block of known weight of each P-rich and C-rich diet. Individuals were kept for three days to accustom them to artificial diets, and to obtain a baseline P:C ratio preference. Thereafter, food blocks were collected, placed at 50 °C until completely dry, and then their weight loss was determined, equating to the amount eaten by the cockroach. Experimental cockroaches were assigned randomly to one of the following sublethal treatments (40 per treatment): 1) High infection (injected 5.8 x 10^5 *P. entomophila* CFUs); 2) Low infection (injected 5.8 x 10^3 *P. entomophila* CFUs); 3) Wounding control (injected Ringer's solution); 4) Unmanipulated control. Cockroaches were then placed on new food blocks of both diets of known weight. The blocks were replaced daily for four days and their loss of weight was again determined after drying at 50 °C.

Survival on enforced diet

From each of 3 cohorts, 10 *B. orientalis* males were placed on P-rich diet (35 % P; 7 % C) and another 10 were placed on C-rich diet (7 % P; 35 % C). All individuals were supplied with water *ad libitum*. Survival was checked twice weekly; food blocks and water were changed once a week over the period of 150 days.

Survival on enforced diet following lethal infection

One hundred and fifty *B. orientalis* males were assigned to one of the following treatments: 1) 90 individuals: Infection (injected 4.0 x 10^6 *P. entomophila* CFUs); 2) 30 individuals: Wounding control (injected Ringer's solution); 3) 30 individuals: Unmanipulated control. A third of the individuals from each treatment were randomly assigned to either a P- (35 % P; 7 % C), C-enriched (7 % P; 35 % C) or a B (21 % P; 21 % C) artificial diet and supplied with water *ad libitum*. Survival of each individual was recorded for 60 h.

Hemolymph collection

Hemolymph for the bacterial growth inhibition assay and proteomic analysis (below) was collected by cutting the first 2 leg pairs and placing the cockroach head-first into a spin-column (Sigma-Aldrich) in a 1.5 ml tube containing propylthiouracil (to inhibit phenol-oxidase activity). They were then centrifuged at 500 *g* for up to 5 min or until 10 µl of hemolymph was collected.

Bacteria growth inhibition assay

One hundred eighty *B. orientalis* males were equally assigned to the following treatments: 1) immune challenged (injected 5.8 x 10^5 *P. entomophila* CFUs); 2) Wounding control (injected Ringer's solution); 3) Unmanipulated control. A third of the individuals from each treatment was randomly assigned to either a P- (35 % P; 7 % C), C-enriched (7 % P; 35 % C) or a B (21 % P; 21 % C) artificial diet and supplied with water *ad libitum*. After 24 h the hemolymph of each individual was collected and 10 µl hemolymph each from 5 individuals was pooled per treatment (resulting in 4 pools per treatment). Those pools were stored at -70 °C till needed.

Bacterial growth inhibition of the cockroach hemolymph was measured using a plate reader assay. First, 10 µl Mueller-Hinton broth were added to each well of a 384-well polypropylene plate. Then 10 µl hemolymph was loaded in the second and the ninth column of the plate. One of these wells contained the hemolymph of one pool of animals (in total 36 wells loaded with hemolymph). Four wells in the first column which did not contain hemolymph served as the negative control. A five-step serial dilution of the hemolymph was performed (with the last 10 µl being discarded) and 10 µl *P. entomophila* in Mueller-Hinton broth with an OD_{600} of 0.005 was added to each well containing hemolymph as well as to another four wells in the ninth

column not containing hemolymph, which served as a positive control for unsuppressed bacterial growth. OD_{600} was measured in a plate reader (BioTek) every 10 min for 16 h.

Proteomic analysis by mass spectrometry

One hundred and twenty *B. orientalis* males were immune challenged by injecting 2 µl Ringer's solution containing 5.8 x 10^5 *P. entomophila* CFUs. Half were assigned to the P- (35 % P; 7 % C) and the other half to the C-enriched (7 % P; 35 % C) artificial diet and supplied with water *ad libitum*. Twenty-four h later the hemolymph of each individual was collected and 10 µl hemolymph each from 10 individuals was pooled per treatment (resulting in 6 pools per treatment). Those pools were stored at -70 °C till needed.

Sample preparation

The protein content of hemolymph was determined using the BCA Protein Assay Kit (Pierce™, Thermo Scientific™). In total, 18 µl of denaturation buffer (Urea 6 M, Thiourea 2 M and HEPES 10 mM) were added to 2 µl of hemolymph samples (this corresponds approximately to 50-60 µg of total protein) and used for in-solution protein digestion (Sury *et al.* 2015). Protein mixtures solubilized in denaturation buffer were reduced with 10 mM dithiothreitol and then alkylated with 55 mM iodoacetamide for 30 min each. A pre-digestion with lysyl endopeptidase (LysC, Wako) was carried out overnight using a proportion of 1 µg enzyme per every 50 µg of protein sample. After pre-digestion with LysC, the samples were diluted fourfold with 50 mM ammonium bicarbonate and subjected to overnight trypsin digestion using 1 µg of sequencing-grade modified trypsin (Promega). All in-solution protein digestion steps were performed at room temperature. The reactions were stopped by adding an equal volume of Buffer A* (5 % acetonitrile, 3 % trifluoroacetic acid). Samples were then desalted and stored using self-made StageTips (Rappsilber *et al.* 2007).

Liquid chromatography-mass spectrometry (LC-MS)

Peptides were reconstituted in 15 µl of 0.1 % trifluoroacetic acid, 5 % acetonitrile and 2 µl were analysed by a reversed-phase capillary nano liquid chromatography system (Ultimate 3000, Thermo Scientific) connected to an Orbitrap Velos mass spectrometer (Thermo Scientific). Liquid chromatography separations were performed on a capillary column (Acclaim

PepMap100 C18, 2 µm, 100 Å, 75 µm i.d. × 25 cm, Thermo Scientific) at an eluent flow rate of 300 nl/min using a gradient of 3-50 % B in 50 min. Mobile phase A contained 0.1 % formic acid in water, and mobile phase B contained 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode utilizing a single mass spectrometry (MS) survey scan (m/z 350-1500) with a resolution of 60,000 in the Orbitrap, and MS/MS scans by collision-induced dissociation of the 20 most intense precursor ions in the linear trap quadrupole.

Proteomic database preparation by de novo transcriptome sequencing

Whole bodies of 8 adult cockroaches were injected through the cuticle with 5 x 10^6 CFUs/g of a cocktail of heat-killed microbes (*P. entomophila, Bacillus thuringiensis, Saccharomyces cerevisiae*) and, as a control, an equal number of cockroaches were injected with Ringer's solution. Both immune challenged and control individuals were used for total RNA extraction, described briefly as follows. Individuals were cut into 4-6 pieces with sterile scissors and RNA was extracted separately before being pooled. Each piece was suspended in pre-cooled Trizol (Thermo Fisher Scientific) and homogenized twice at 4 m/s for 15 s using a homogenizer (FastPrep™-24, MP Biomedicals) with a 5 mm stainless steel bead (Qiagen). Recovery of RNA was achieved following manufacturer's instructions, using chloroform extraction and isopropanol precipitation, and re-dissolution in RNA storage solution (Ambion). Subsequently, samples were incubated with 2 units of TurboDNase (Ambion) for 30 min at 37 °C and RNA was purified using RNAeasy Mini kit (Qiagen) according to manufacturer's instructions. Quantity and quality of RNA were determined by Qubit 2.0 and Bioanalyzer 2100. Equal amounts of total RNA from 4 individual extractions were pooled together to form 4 libraries. Subsequently, the mRNA libraries were enriched and prepared using a NEXTflexTM Rapid Directional mRNA-seq Kit protocol (Bioo Scientific). The prepared libraries were sequenced on an Illumina NextSeq500/550 platform at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv).

The raw data were analysed as described elsewhere (He *et al.* 2018). Briefly, reads were trimmed and filtered using Trimmomatic, as incorporated in Trinity v2.2.0 (Grabherr *et al.* 2011). Paired-end reads were assembled using Trinity with default parameters (Kmer size: 25) on a local server. The transcriptome was annotated following the guidelines of Trinotate (https://trinotate.github.io/). Protein domains, signal peptides, and transmembrane domains were determined by HMMER v3.1b2 against the pfam database (Finn *et al.* 2011), SignalP v4.0 (Petersen *et al.* 2011), and TMHMM 2.0 (Krogh *et al.* 2001), respectively. Homology searches, predictions and domain identifications were performed locally and subsequently

integrated into SQLite database with SQLite v3.11.0 at an e-value threshold of $1e^{-03}$. The highest expressed isoforms from each contig were filtered for following proteomic data analysis.

Proteomic data analysis

Identification and label-free quantification of proteins was performed using the freely available software suit MaxQuant v1.6.0.1 with implemented Andromeda search engine (Cox and Mann 2008; Cox *et al.* 2011; Tyanova *et al.* 2015). Raw data were matched against an in-house protein database of *B. orientalis* created by *de novo* transcriptome sequencing (see above). Trypsin was selected as enzyme and a maximum of two missed cleavages was allowed. Cysteine carbamidomethylation was set as a fixed modification. Methionine oxidation and acetylation (protein N-terminus) were allowed as variable modifications. The 'match between runs option' was used using a 0.7 min match time window and a 20 min alignment time window. The option to report iBAQ values was activated (Schwanhäusser *et al.* 2011). The minimum peptide length was set to 7 amino acids and the false discovery rate (FDR) for peptide and protein identification was set to 0.01.

Proteomic data processing and statistical analysis

Data processing and statistical analysis was performed with PERSEUS software v1.6.0.2 (Tyanova *et al.* 2016; Tyanova and Cox 2018). Proteins identified from the contaminant database, reverse hits and proteins only identified by site were removed as well as proteins with less than three valid values. For the remaining 387 proteins imputation of missing values was performed (width 0.3, down shift 1.8). Significant changes in protein abundance between the two treatments were calculated by a student's t-test using permutation-based FDR of 0.05. Only proteins with significant abundance changes ($q < 0.05$) were considered. In addition, a minimum 2-fold change in protein abundance was set as a threshold.

After peptides from the LC-MS/MS analysis were matched to our transcriptome-derived predicted protein database, we ascertained the putative functions of proteins by querying the annotated functional database associated with our transcriptome-derived predicted-protein database. The functional annotation of the majority of these proteins was derived from SwissProt queries using Blastp (UniProt Consortium 2018) and proteins that could not be identified using Blastp were assigned using HMMER search results from the annotated

functional database. Proteins that could not be annotated with Trinotate suit tools (Blastp, HMMER) were assigned ‘unknown’ function.

Statistical analysis

P:C ratios, the amounts of P and C eaten as well as total consumption differences between treatments for the first day following infection were analysed using Bonferroni-corrected Wilcoxon rank sum tests.

The food-choice data were analysed using a generalized linear mixed model (GLMM) with an underlying beta family distribution. Analyses were run in the glmmADMB package v0.8.3.3 (Fournier *et al.* 2012; Skaug *et al.* 2014) in R 3.6.1 (R Core Team 2019) in conjunction with the R2admb package v0.7.16 (Bolker *et al.* 2017). GLMMs examined whether a response variable consisting of proportion of P consumed (amount of P eaten divided by the amount of diet available) or proportion of C (amount of C eaten divided by the amount of diet available) was influenced by treatment (high infection; low infection; wounded; and unmanipulated) and day post infection as well as an interaction between treatment and day. Minimal adequate models were derived by stepwise-model simplification and comparison via ANOVA. Individual and cohort were treated as random effects to account for multiple measurements and origin. Comparisons among treatment levels were carried out with post-hoc Tukey tests using a Bonferroni correction, using package multcomp v1.4-10 (Hothorn *et al.* 2008). Individuals 16, 48 82, 84 and 91 were removed prior to analysis due to the presence of fungal growth on the artificial diet blocks.

The effect of treatment and diet on survival was analysed using Cox proportional hazard models in R 3.6.1 (R Core Team 2019) with the survival package v2.44-1.1 (Therneau 2015). Because control data in the survival following infection experiment were right-censored, we uncensored one randomly selected individual from each treatment, following Tragust *et al.* (2013). Median survival time for each treatment was calculated using the survminer package v0.4.6 (Kassambara *et al.* 2019). Pairwise comparisons among treatment levels were carried out using post-hoc Tukey tests with a Bonferroni correction, using the package multcomp v1.4-10 (Hothorn *et al.* 2008). Bacterial growth inhibition data were analysed using R package nparLD v2.1 (Noguchi *et al.* 2012) using default parameters for pairwise comparisons.

Results

Survival on enforced diet without infection

The median (50 %) survival time for *B. orientalis* males placed on a P-rich diet was 82 days, whereas the mortality of males placed on a C-rich diet did not exceed 30 % over the course of the experiment (150 days; Fig. 2.2A). By the end of the experiment males restricted to P-rich diet showed a significantly higher mortality (86.44 %) compared to those on C-rich diet (27.59 %; Cox proportional hazard regression P vs. C: $z = 5.974$, $p < 0.001$).

Diet choice following sublethal infection

Before wounding or infection, cockroaches of all treatments preferred an average P:C ratio of approximately 0.28 (Fig. 2.1A). The unmanipulated animals preferred this ratio over the course of the experiment. By contrast, highly infected individuals selected a P:C ratio of approximately 1.3 whereas low infected and wounded cockroaches preferred an intermediate ratio of approximately 1.0 P:C on the first day post-infection (p.i.; Wilcoxon rank sum test: high vs. low: $W = 595.0$, $p = 0.098$; low vs. wounded: $W = 412.5$, $p > 0.1$; wounded vs. unmanipulated: $W = 713.5$, $p < 0.001$). All manipulated groups returned to baseline P:C ratios by day 4 p.i..

Final minimal GLMMs consisted of the fixed terms treatment and day without an interaction since the model with a treatment*day interaction did not significantly improve the model (ANOVA for model comparison, $p > 0.1$). Cockroaches that were wounded differed significantly from unmanipulated cockroaches in their preferred P proportion on the first day following treatment (P proportion chosen: 0.14 vs. 0.06, wounded vs. unmanipulated, respectively: $z = -3.348$ $p = 0.005$), as did cockroaches infected with a high (P proportion chosen: 0.17 vs. 0.06, high vs. unmanipulated respectively: $z = -7.416$, $p < 0.001$) or low bacterial dose (P proportion chosen: 0.14 vs 0.06, low vs. unmanipulated respectively: $z = -2.809$, $p = 0.029$). Cockroaches infected with a high bacterial dose also selected a higher proportion of P compared to individuals exposed to both a low bacterial dose (P proportion chosen: 0.17 vs. 0.14, high vs. low respectively: $z = -3.718$, $p = 0.001$) or to wounding (P proportion chosen: 0.17 vs. 0.14, high vs. wounded respectively: $z = -2.808$, $p = 0.029$). However, individuals that were wounded or were infected with a low bacterial dose did not select a significantly different proportion of P (P proportion chosen: 0.14 vs. 0.14, low vs. wounded respectively: $z = 0.625$, $p = 1.000$). Concerning the proportion of C, the pattern is slightly different. Only highly infected

cockroaches on the first day p.i. differed significantly from unmanipulated (C proportion chosen: 0.16 vs. 0.26, high vs. unmanipulated respectively: z = 5.270, $p < 0.001$). All the other treatments were not significantly different from each other (Supplementary Tab. 2).

P, C as well as total food consumption varied significantly over the course of the experiment (Fig. 2.1B-D). Individual cockroaches ate on average 0.6 mg P and 2.2 mg C under unmanipulated conditions. Conversely, in all manipulated groups the amount of P eaten declined immediately after infection (Wilcoxon rank sum test: wounded vs. unmanipulated: W = 77.0, p < 0.001; low vs. unmanipulated: W = 66.5, p < 0.001; high vs. unmanipulated: W = 76.0, p < 0.001). The same accounts for the consumption of C (Wilcoxon rank sum test: wounded vs. unmanipulated: W = 11.0, p < 0.001; low vs. unmanipulated: W = 7.0, p < 0.001; high vs. unmanipulated: W = 6.0, p < 0.001). As a result, the total amount eaten was reduced in the same way immediately following treatment (Wilcoxon rank sum test: wounded vs. unmanipulated: W = 0.0, p < 0.001; low vs. unmanipulated: W = 0.0, p < 0.001; high vs. unmanipulated: W = 0.0, p < 0.001). By the 2nd day, consumption across all groups began to recover, reaching pre-treatment levels by the 4th day p.i..

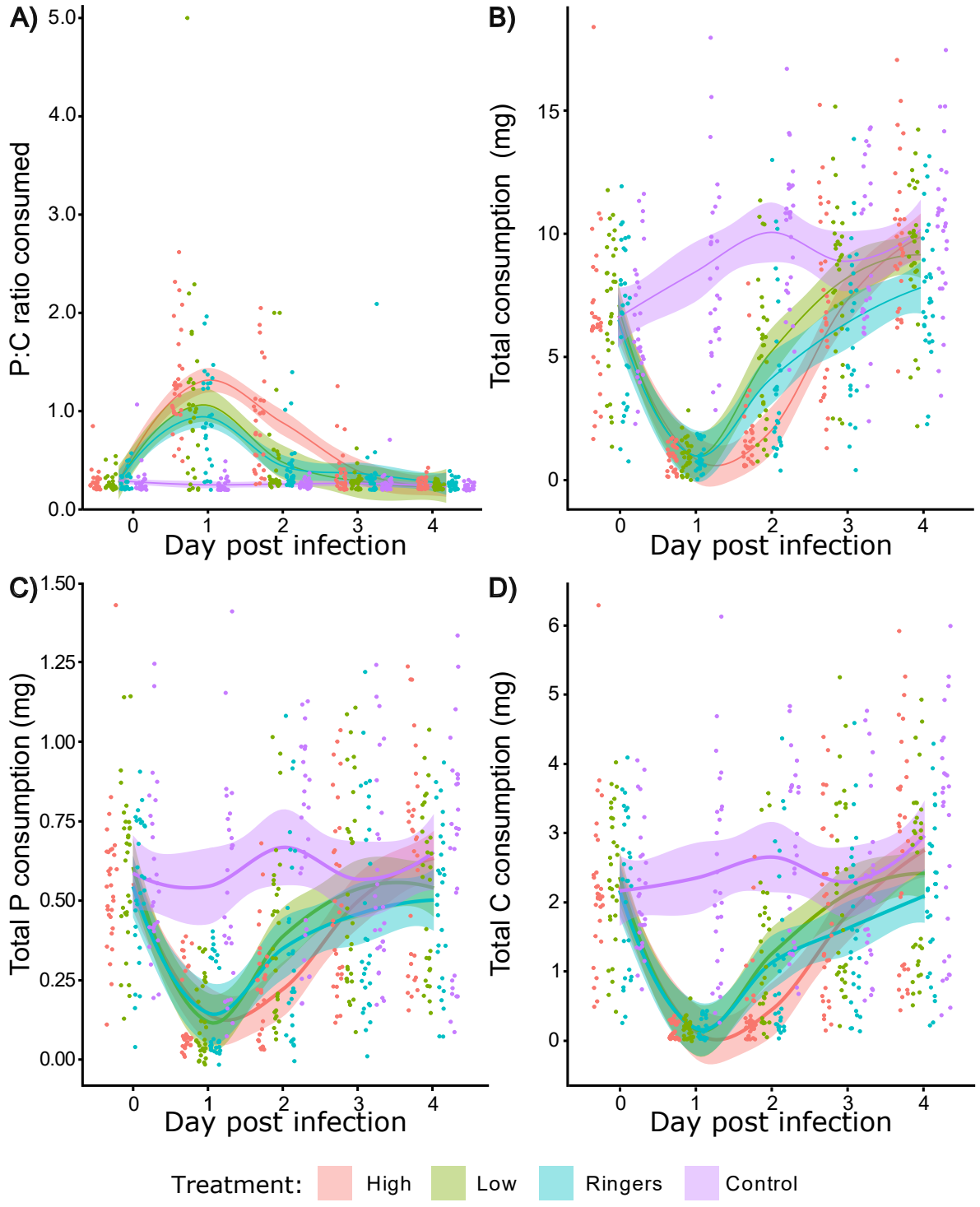

Fig. 2.1: Effect of bacterial infection with *P. entomophila* (high load, low load), Ringer's solution or no manipulation (control) of *B. orientalis* males on: **A)** P:C ratio chosen, **B)** total consumption, **C)** P consumption, **D)** C consumption. Note different scales used for total P and C consumption. Area surrounding mean curves indicate 95 % confidence intervals.

Survival on enforced diet following infection

In our test of the effect of dietary composition on survival following lethal infection, we found that cockroaches on all diets began to die at 20 h after injection (Fig. 2.2B). This included individuals on the B diet, which most closely resembled the ratio selected by cockroaches following sublethal infection. The median survival time for infected *B. orientalis* males was 26, 25 and 27 h on P-rich, C-rich and B diets, but the effect of diet on survival was not significant (Cox proportional hazard regression: $P_{infected}$ vs. $C_{infected}$: z = 1.961, p = 1.000; $B_{infected}$ vs. $C_{infected}$: z = 1.764, p = 1.000; $B_{infected}$ vs. $P_{infected}$: z = -0.247 , p = 1.000). No mortality occurred during the course of the experiment in the wounded and unmanipulated *B. orientalis* males, independent of dietary treatment.

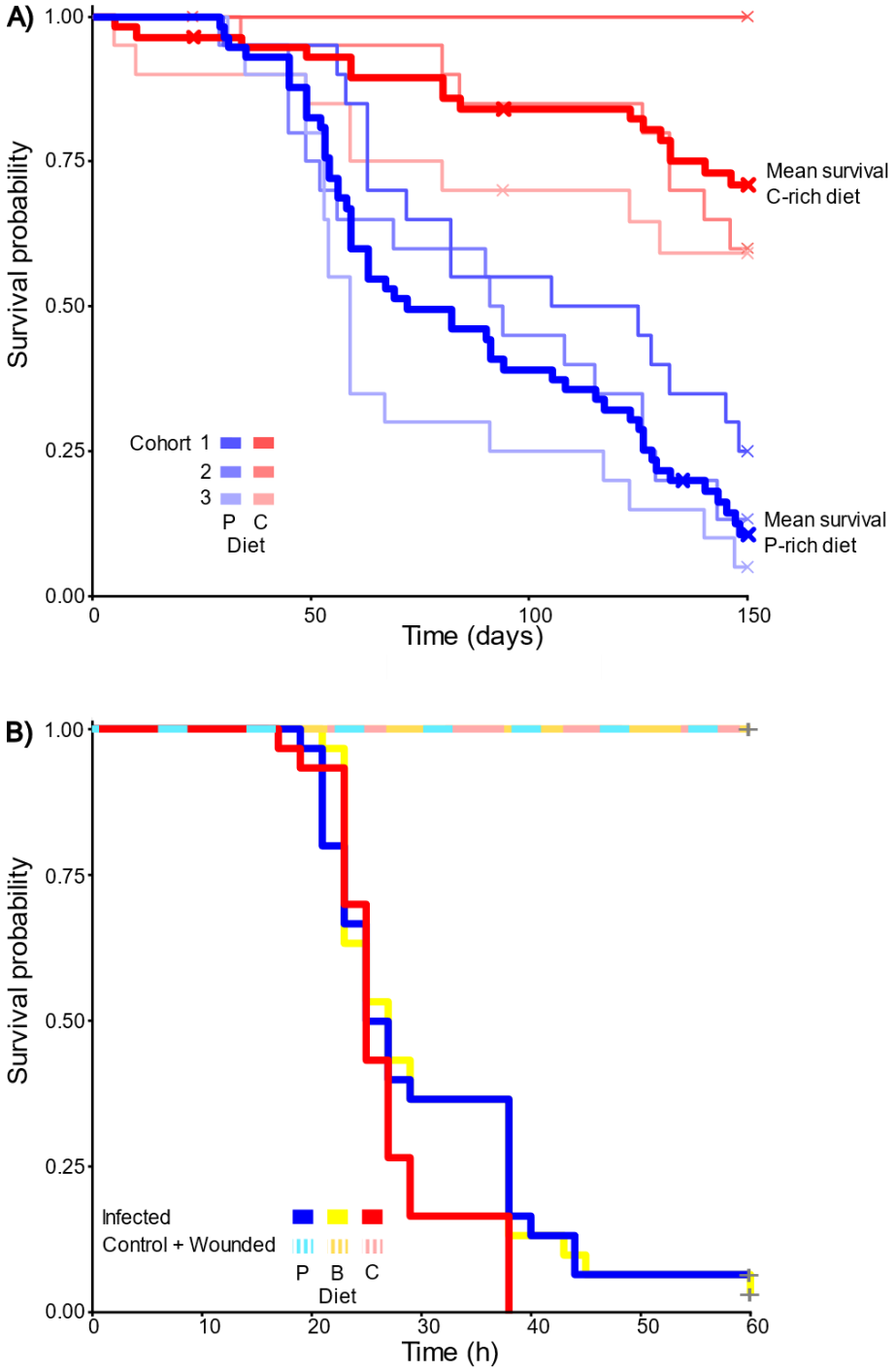

Fig. 2.2: Kaplan-Meier survival curves of: **A)** Unmanipulated *B. orientalis* males restricted to P-rich (35 % P and 7 % C) or C-rich (7 % P and 35 % C) diets. Survival data for three independent cohorts (1-3) for P- and C-rich diets are given in blue and red respectively, with mean population survival across cohorts on each diet indicated by a thick bold line. Note the long period at the beginning of the experiment where no clear survival differences between diets are observable. **B)** *B. orientalis* males restricted to P-rich (35 % P and 7 % C; blue lines), C-rich (7 % P and 35 % C; red lines), or B (21 % P and 21 % C; yellow lines) diet following

injection with an LD_{50} of *P. entomophila* (infected), Ringer's solution (wounded) or being unmanipulated (control).

Bacteria growth inhibition assay

The inhibitory effect of male *B. orientalis* hemolymph (N = 4 per dilution per treatment) on bacterial growth was not diet-dependent, regardless of treatment (immune challenged, wounded or unmanipulated; Fig. 2.3). This resulted in similar suppression of bacterial growth regardless of diet (Group effect: $P_{infected}$ vs. $C_{infected}$: df = 1, $p > 0.1$; $B_{infected}$ vs. $C_{infected}$: df = 1, $p > 0.1$; $B_{infected}$ vs. $P_{infected}$: df = 1 , $p > 0.1$; $P_{wounded}$ vs. $C_{wounded}$: df = 1, $p > 0.1$; $B_{wounded}$ vs. $C_{wounded}$: df = 1, $p > 0.1$; $B_{wounded}$ vs. $P_{wounded}$: df = 1 , $p > 0.1$; $P_{unmanipulated}$ vs. $C_{unmanipulated}$: df = 1, $p > 0.1$; $B_{unmanipulated}$ vs. $C_{unmanipulated}$: df = 1, $p > 0.1$; $B_{unmanipulated}$ vs. $P_{unmanipulated}$: df = 1 , $p > 0.1$. Nevertheless, all treatments were significantly different to both the negative and the positive controls (Supplementary Tab. 3). Additional comparisons which are not listed including the time effect and the interaction of time and group are listed in Supplementary Tab. 3 as well.

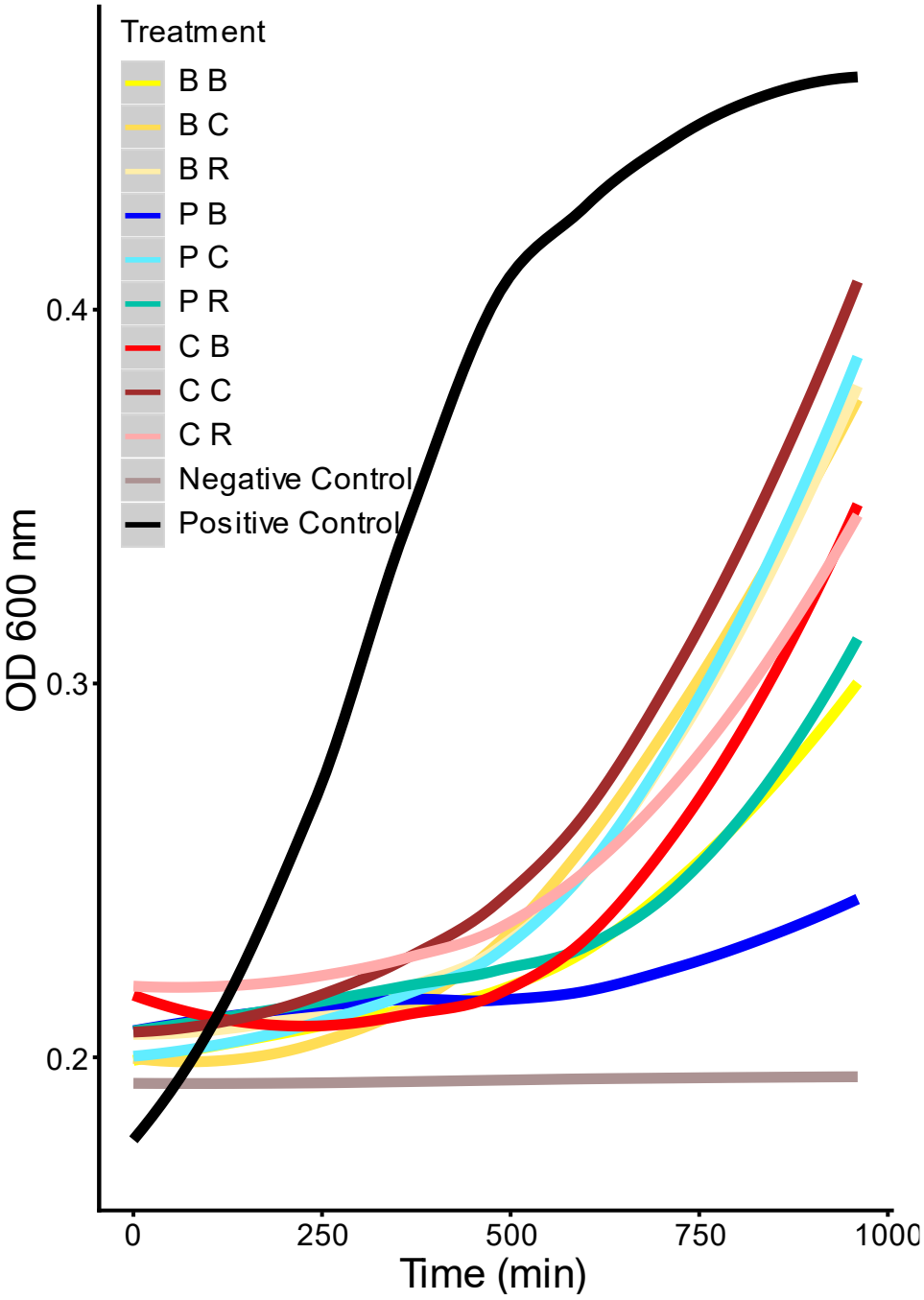

Fig. 2.3: Impact of diet on *B. orientalis* hemolymph growth inhibition of *P. entomophila in vitro* (1:4 dilution). Immune challenged individuals on P-rich (P B), C-rich (C B) or B (B B) diet. Ringer's solution injected (wounded) individuals on P-rich (P R), C-rich (C R) or B (B R) diet. Control (untreated) individuals on P-rich (P C), C-rich (C C) or B (B C) diet. A bacterial solution without hemolymph served as the positive control and a solution containing only the growth medium (Mueller Hinton) served as the negative control.

Proteomic analysis by mass spectrometry

We were unable to detect any effect of a B diet on either cockroach survival or hemolymph antimicrobial activity, regardless of infection treatment, and so restricted our proteomic analysis to a comparison of P-rich and C-rich diets following sublethal challenge. A total number of 3514 peptide hits were identified and assembled into 750 proteins by MaxQuant. After filtering, 387 different proteins were identified and quantified in the hemolymph of infected *B. orientalis* males fed on a P-rich vs. a C-rich diet (N = 6 per treatment) (Supplementary Tab. 4), of which 65 are putative immune-related genes. Overall, apolipophorin was the most abundant protein making up approximately 70 % of the whole hemolymph protein content. Other highly abundant proteins were transferrin, gelsolin, heterochromatin-associated protein MENT and an insulin-like growth factor-binding protein complex. We identified 17 proteins that showed significant changes in abundance following diet treatment (Fig. 2.4 and Supplementary Tab. 4). Infected individuals on a C-rich diet were significantly enriched for hexokinase type II, which is involved in carbohydrate metabolism (glycolysis; Yanagawa 1978), in addition to carbonyl reductase I-like, which is involved in NADPH-dependent reduction of active substrates including endogenous and xenobiotic carbonyl compounds (Hoffmann and Maser 2007). Additionally, tropomyosin which is a calcium-dependent regulator of muscle contraction (Pomés *et al.* 2007), and acyl-CoA-binding protein, which carries out lipid-binding transport and suppresses glucose-induced insulin secretion (Færgeman *et al.* 2007) were more abundant. Furthermore, a L-galactose dehydrogenase-like protein was enriched but its function is not known in insects. Conversely infected individuals on a P-rich diet were significantly enriched for alpha-amylase, which is involved in carbohydrate metabolism (Terra and Ferreira 1994) and proteasome subunit alpha type-3, which is involved in protein degradation (Rivett 1993). Additionally, hemolymph lipopolysaccharide (lps)-binding protein-like (2 isoforms), which binds carbohydrates (foreign particles; Jomori and Natori 1991) and extracellular superoxide dismutase, which carries out superoxide metabolic processing (Felton and Summers 1995) were detected. Glutamine synthetase is involved in glutamate and glutamine catabolism and biosynthesis (Smartt *et al.* 1998) while adenylate kinase isoenzyme 1 and hexamerin are associated with ATP metabolism (Fujisawa *et al.* 2009) and amino acid and energy storage, respectively (Burmester 1999). There was also an enrichment of ankyrin-1, although its function in insects remains unclear.

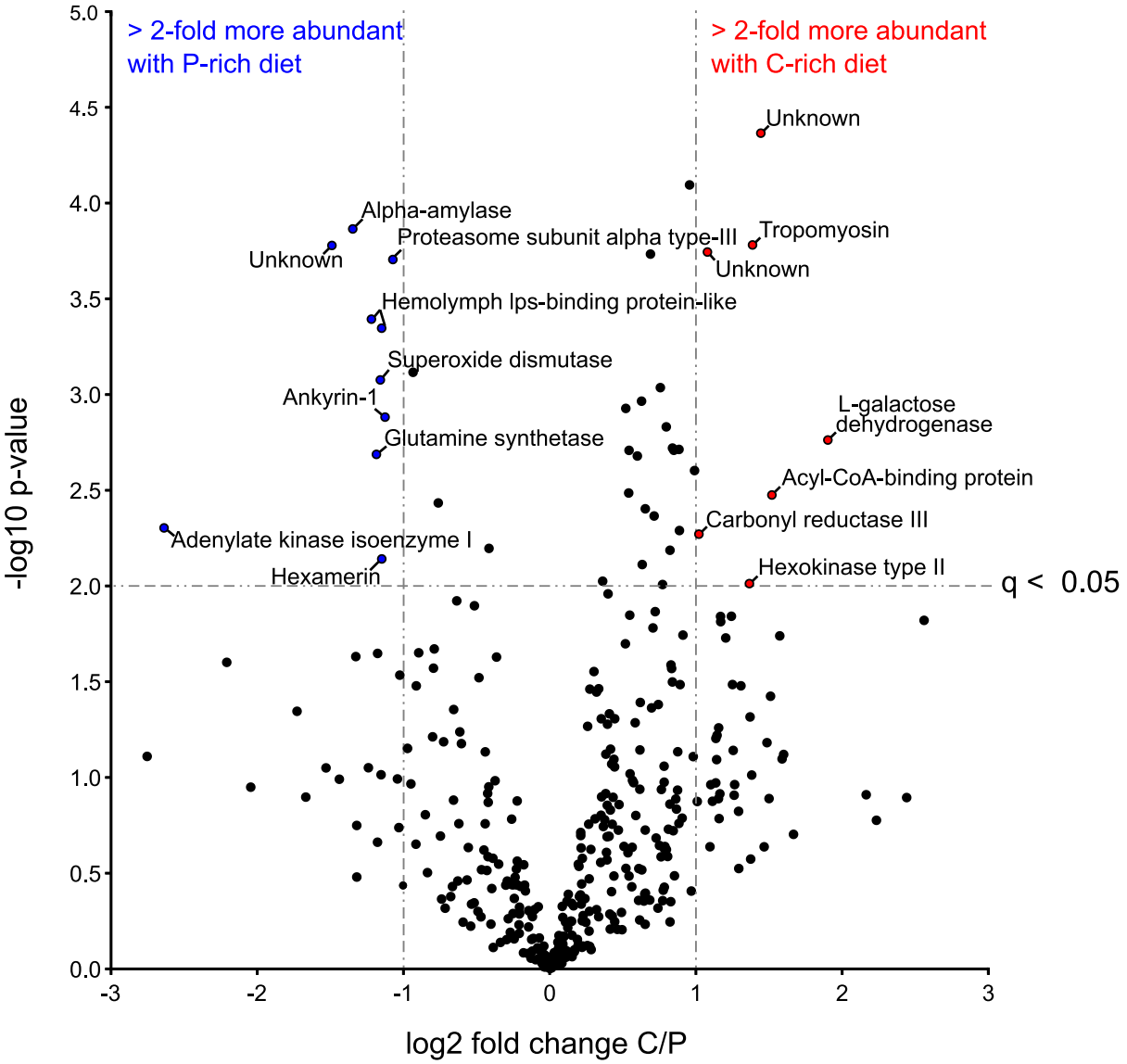

Fig. 2.4: Effect of diet on abundance of male *B. orientalis* hemolymph proteins following immune challenge (high dose). Points in blue and red reflect proteins that are significantly (> 2) more abundant in P- and C-rich diets, respectively.

Discussion

Under normal conditions, extensive P consumption shortens the lifespan of many insects including ants, honeybees and flies (Lee *et al.* 2008b; Dussutour and Simpson 2009; Fanson *et al.* 2009; Grandison *et al.* 2009; Cook *et al.* 2010; Pirk *et al.* 2010), a finding that is corroborated in our and another study of cockroaches (Hamilton *et al.* 1990). Here, we find that male *B. orientalis* cockroaches showed 45 % higher mortality (Fig. 2.2A) when restricted to a P- vs. a C-rich diet. One explanation for this consistent observation across study

organisms is that elevated levels of P increase TOR signalling. TOR serves as a nutrient sensor linked to macronutrient intake and metabolism, causing a broad anabolic response that is life-shortening over the long term (reviewed in Simpson and Raubenheimer 2009). Other explanations could relate to the toxic effects of breaking down nitrogenous products, and the enhanced production of mitochondrial radical oxygen species, DNA and protein oxidative modifications, membrane fatty acid composition and mitochondrial metabolism (reviewed in Simpson and Raubenheimer 2009). The higher abundance of extracellular superoxide dismutase in cockroach males fed on a P-rich diet (Fig. 2.4; Supplementary Tab. 4) supports this explanation.

Unsurprisingly, male cockroaches consumed low amounts of P under normal conditions. But this preference shifted dramatically following infection. As with caterpillars (Povey *et al.* 2013), highly infected male cockroaches switched to a P-rich diet. Furthermore, cockroaches adapted their feeding behaviour to the severity of the infection. Lowly infected and wounded (Ringer-injected) individuals chose an intermediate (balanced) P:C ratio, whereas uninfected and highly infected individuals chose a significantly enriched diet for C and P, respectively. This demonstrates that cockroaches quantitatively regulate their behavioural response to infection. Additionally, our data suggest host-driven adaptation as opposed to pathogen manipulation because wounded individuals also reduced their C intake. Wounding elicits a localized immune response in insects (Haine *et al.* 2007), suggesting a form of prophylactic behaviour since it is likely that microbes can enter the hemolymph via damaged cuticle (Siva-Jothy *et al.* 2005).

In contrast to caterpillars and other organisms which can modulate their immune response with diet, changes in cockroach dietary preference did not greatly influence any of the immune parameters we measured. In caterpillars, a shift from a C- to a P-biased diet following *B. subtilis* (Gram-positive) or baculovirus infection led to an increase of antibacterial and PO activity and hemocyte density, and resulted in higher survival (Povey *et al.* 2009; Povey *et al.* 2013). By contrast, a switch to a P-biased diet did not significantly influence cockroach hemolymph antimicrobial activity or survival, nor have a major impact on the production of immune-related proteins. Only two hemolymph lps-binding protein isoforms, which may play a role in pathogen recognition by binding foreign particles (Jomori and Natori 1991) were more abundant in the hemolymph of P-rich fed infected cockroaches, but overall our findings indicate that the behavioural changes adopted by cockroaches are limited in their ability to alter infection outcome.

The overrepresentation of proteins participating in carbohydrate and protein metabolism in C- vs. P-rich diets, respectively (Fig. 2.4), demonstrate that the diets altered cockroach physiology in the expected direction. For example, the higher abundance of alpha-amylase in the

hemolymph of *B. orientalis* males feeding on P-rich diet shows these individuals were metabolizing lower quantities of C. Alpha-amylase is thought to be involved in the breakdown of glycogen, which is the major glucose storage compound in animals. It is employed if not enough C is present in the diet (Mohamed 2004).

Overall, our results suggest that cockroaches may not be able to self-medicate using macronutrients, but that they do engage in a typical anorexia response, as has been shown in macronutrient self-medication in caterpillars (Adamo *et al.* 2007; Povey *et al.* 2009; Povey *et al.* 2013). Illness-induced anorexia offsets physiological trade-offs between launching immune responses and food digestion. A previous study demonstrated that crickets reduce their food intake, especially for lipids, following infection with the bacterium *Serratia marcescens* (Adamo *et al.* 2010). High hemolymph lipid levels are associated with decreased concentrations of monomeric apolipophorin III, a lipid transporter, and higher susceptibility to *S. marcescens* infection (Adamo *et al.* 2008). In other insects, anorexia can have a direct impact on immunity. For example, in *Drosophila*, starvation can modify AMP production and lead to reduced melanisation (Ayres and Schneider 2009).

The apparent lack of a link between macronutrient dietary selection and male cockroach immunity is unexpected. One possible explanation is that future food availability and quality may be far less predictable in omnivorous pest organisms like cockroaches (Raubenheimer and Jones 2006). A recent genomic study reports major expansions of cockroach gene families linked to chemoreception, detoxification and innate immunity (Li *et al.* 2018), indicating that adaptations in these pathways may have been essential for enabling cockroaches to thrive in unpredictable, antigen-rich environments. Indeed, while cockroach survival was reduced on an enforced P-rich diet, a negative effect could only be observed well over 40 days after exposure. In such organisms, there could be an advantage in reducing regulatory interactions between host diet and immunity. An additional point to consider is that in contrast to several previous studies, we performed experiments on adult individuals and not larvae, which have different resource allocation strategies and consumption rates in general (Boggs 2009). In holometabolous insects, most of the resources in larvae are allocated to growth, maintenance and storage whereas in adults, they are allocated to reproduction and maintenance. Consequently, there has been great emphasis on a trade-off between growth and immunity in the larval stage of herbivorous insects (reviewed in Singer *et al.* 2014). Here, the need for fast growth competes with the requirement to provide protection from parasite and pathogen-induced mortality. Given that P and amino acids are a crucial limiting factor in herbivorous diets, we argue that a trade-off between two essential life-history parameters that depend strongly on P – growth and survival – could be particularly pronounced in herbivorous insects (Schoonhoven *et al.* 2005; Simpson and Raubenheimer 2012).

In conclusion, we find that *B. orientalis* males modulate their macronutrient feeding behaviour following infection by dramatically reducing food intake and favouring a diet containing a higher P ratio. We also show that a P-rich diet eventually leads to significantly reduced host lifespan, and that male cockroaches avoid such a diet under normal conditions. To our surprise, the observed behavioural response to immune challenge did not meaningfully influence the antimicrobial activity or proteomic profile of host immunity. Our findings support the concept of a generalized host-directed response to microbial challenge in cockroaches based on anorexia and the limitation of C intake. Such a response may be beneficial to the host, but perhaps primarily as a means of avoiding contaminated food and reducing pathogen access to resources, rather than facilitating crosstalk with the immune system. This could be the result of adaptations to detoxification and innate immunity to survival in antigen-rich and nutritionally diverse environments, although this hypothesis requires additional testing. Overall, our study highlights the importance of understanding variation in natural diet, development, and ecology when exploring the link between nutrition and animal immunity.

Chapter II

Cleaning to a higher standard: evaluating sterilization techniques for the creation of a germ-free *Blatella germanica* breeding line and the impact of the microbiome on host development

Abstract

1. Depriving organisms throughout their lifetime of their natural microbiota, i.e. producing germ-free individuals, is an effective way to gain insights into the contribution of microbiota on host traits like development, physiology and immunity. Insects are especially suitable for these kinds of studies because of their short lifecycle, their large numbers of offspring, the relatively few microbial species they are inhabited by and the vast amount of cheap and easy to apply sterilization techniques.
2. However, these manifold sterilization techniques also present a problem – there are so many different techniques available, that finding the most efficient one for a certain species is quite a complicated task. For *B. germanica* cockroaches there are for example at least two different surface sterilization methods already described. While one uses peracetic acid, the other one uses sodium hypochlorite. It is worth mentioning that both methods were established before the age of 16S rDNA sequencing and therefore their effectiveness was so far only estimated by microbial growth assays.
3. Here, we therefore tried to establish an effective sterilization method for *B. germanica* to create individuals mostly only inhabited by their obligate symbiont *Blattabacterium sp*.. To achieve this, we first tested the success of a peracetic treatment alone. We further combined it with a sodium hypochlorite and later also with an additional antibiotic treatment with rifampicin and gentamicin of freshly hatched nymphs. We examined the success of our different treatments by plating adult cockroaches on LB-agar and additionally tested both surface sterilization techniques via 16S rDNA sequencing. Furthermore, we used sterilised cockroaches for developmental studies where we compared them to conventional ones to gain more insights on the impacts of the microbiota in this species and to support some old studies which found that generally germ-free individuals needed longer to molt into adults.
4. We found that independent of the method, surface sterilization alone (without the use of antibiotics) leads to sufficiently sterile cockroaches in 40 % of all cases already proved by examining the microbial growth by plating. Anyhow, any setup which was tested sterile by plating also showed no or only rudimentary microbial abundance by 16S rDNA sequencing. Notably, the additional antibiotic treatment improved the effectiveness of the sterilization to 99 % already indicated by plating. Furthermore, regarding the developmental time experiment we found that depriving individuals of their natural microbiota meant that they needed approximately 35 days longer from the day of hatching to the day of molting into adults than their conventional counterparts which needed approximately 43 days in total.

5. We show that even if there are already proven sterilization methods available in the literature, it is always necessary to check their effectiveness and sometimes it is also possible to further improve them. Moreover, we could show the microbiota impacts the development and therefore also the physiology of *B. germanica* in a huge way and combining this with the fact, that microbes not only support development in invertebrates, but also in vertebrates underlines that these host-microbe interactions are very successful.

Keywords: microbiome, germ-free techniques, metabarcoding, development

Introduction

To gain insights into the contribution of the microbiome, also referred to as the 'forgotten organ' (O'Hara and Shanahan 2006), to host traits like development, physiology and immunity the easiest method is to compare individuals experimentally deprived throughout their lifetime of their microbiota (germ-free) with conventional ones bearing their natural microbial community (reviewed in Al-Asmakh and Zadjali 2015; Douglas 2011). The concept of germ-free animals was already recognized and inspired by a debate between Emile Duclaux and Louis Pasteur in 1885 on the question if microbes are mandatory for any higher living organism to live (reviewed in Kirk 2012). Pasteur believed that microbes are mandatory but already 10 years later he was proved wrong by Nuttle and Theirfelder who created the first germ-free guinea pig at the University of Berlin (reviewed in Al-Asmakh and Zadjali 2015). Nevertheless, due to a lack of knowledge concerning nutritional needs, it took 50 years more until the first germ-free rat colony was successfully established by Gustafsson at the University of Lund (reviewed in Al-Asmakh and Zadjali 2015). Since then, experiments on germ-free vertebrates are still technically demanding and, in some cases, hard to interpret because many microorganisms are members of complex consortia with a lot of functional redundancy (Douglas 2011). In contrast, insects are particularly well-suited for this kind of application due to several reasons: 1) their short lifecycle, 2) their high offspring numbers, 3) the variety of cheap and easy to apply sterilization techniques which are available for different insect species and 4) their low numbers of different microbial species, which makes it straight forward to assign functions to single microbial taxa (Douglas 2011).

For insects and other invertebrates, a lot of different techniques have been described to produce germ-free individuals, but not all of them have proved to equally be successful or practical (reviewed in Doll *et al.* 1963). One of the earliest approaches being used was the time-consuming sedimentation trough columns of sterile Labarraque's and Ringer's solution by Glaser and Stoll (1938) for the sterilization of nematodes or only Ringer's solution by Ferguson (1940) for the sterilization of trematodes. Those methods were further improved by others by adding antibiotics to the sedimentation columns (reviewed in Doll *et al.* 1963). The most of the time much faster chemical sterilization (Doll *et al.* 1963) was reported as early as 1917 for *A. aegypti* by Atkin and Bacot (1917) who tested different methods including the chemicals lysol and formalin, followed by many different other methods in various insects in the years after. MacGregor (1929) also tried different reagents on *A. aegypti* until he came up with using 5 % potassium soap solution and an immersion in 80 % ethyl alcohol. In 1959, which seemed to be a quite busy year for germ-free insect rearing, Cheldelin and Newburg (1959) used 2 % sodium hydroxide solution to sterilize brown fly eggs whereas, Waterhouse (1959)

used Clorox and 0.1 % Zephiran for wax moths and Friend *et al.* (1959) created sterile onion maggots (larvae of *Delia antiqua*) by immersing them in 20 % formalin.

Nowadays, there are even more techniques for all kinds of insects available and even the often-overlooked cockroaches have gained quite some attention. Tegtemeier *et al.* (2016) used peracetic acid to surface-sterilize ootheca of the Turkestan cockroach *S. lateralis*, a method which was also used earlier by Doll *et al.* (1963) to obtain germ-free *B. germanica* cockroaches and *D. melanogaster* flies. In contrast, Benschoter and Wrenn (1972) had good results using 0.25 % sodium hypochlorite for ootheca surface sterilization to obtain germ-free *B. germanica* roaches, a technique which was also performed in a comparable manner in the same species by Wada-Katsumata *et al.* (2015) who used 0.5 % sodium hypochlorite and 70 % ethanol.

The various techniques being available to generate germ-free cockroaches have helped to shed light on some possible roles of microbiota (for its general composition see '**General introduction - Cockroaches as a great model to study the tripartite of hosts, their microbiomes and pathogens**') in cockroach biology (Jahnes *et al.* 2019) which are also transferable to other organisms. So far, it is known that in their fat bodies, cockroaches harbour intracellular, maternally transovarially transmitted and unculturable bacteria, which are called *Blattabacterium sp.* (Gier 1936; Sacchi *et al.* 1988; Liesack and Stackebrandt 1992). These Gram-negative bacteria can recycle nitrogen from urea and ammonia into glutamate and they can subsequently produce all essential amino acids and various vitamins (Sabree *et al.* 2009). Regarding other microbiota it is known that that the transmission of feces and in small parts also the diet as well as conspecific coprophagy contribute significantly to its establishment (Rosas *et al.* 2018; Jahnes *et al.* 2019). Furthermore, it was shown that the microbiota of cockroaches produces volatile carboxylic compounds which are emitted in the feces and elicit aggregation behaviour and therefore plays a role in the insect's communication in a similar manner as it does in locusts (Dillon *et al.* 2002; Wada-Katsumata *et al.* 2015). Additionally, it also seems to be involved in the degradation of plant polymers (Hackstein and Stumm 1994; Zurek and Keddie 1998) and in development (Clayton 1959; Bracke *et al.* 1978; Jahnes *et al.* 2019).

Nevertheless, there are many unanswered questions, especially on the impacts of the microbiota on overall cockroach physiology and gene regulation. Additionally, it seems that a general working sterilization technique at least for *B. germanica* still needs to be found, since all the different studies seem not to be able to agree on one established method, pointing to difficulties which still need to be overcome. Therefore, we aimed to further improve the available methods for establishing a germ-free *B. germanica* breeding system, to path the way

for future studies. It is worth noting, that it is mandatory that the germ-free cockroaches still carry their obligate endosymbiont *Blattabacterium sp*., because otherwise they are not able to develop into fertile adults under normal circumstances (Brooks and Richards 1955).

We tested the success of our new methods via plating assays and 16S rDNA sequencing which is typically used to probe bacterial community structure and diversity (Zaneveld *et al.* 2008). Furthermore, we compared the developmental times of conventional and germ-free cockroaches. From other insect species it is already known, that depriving them of their inherent microbiota can drastically delay their development. Depriving, for example, the mosquito *Anopheles stephensi* with rifampicin from its symbiotic acetic acid bacteria (genus *Asaia*) lead to a delayed larval development (Chouaia *et al.* 2012). Additionally, when the stinkbug, *R. pedestris* fails to acquire a *Burkholderia* symbiont orally within a specific developmental window its development is atrophied (Kikuchi *et al.* 2007; Kikuchi *et al.* 2011). Moreover, germ-free *Schistocerca gregaria* desert locusts for example need twice as long to develop into mature adults than conventional ones (Charnley *et al.* 1985). Additionally, studies on germ-free *D. melanogaster* could show that live bacteria speed up the insect's development (Gould *et al.* 2018). On top of that, it is also already known that the microbiota of cockroaches contributes to their development. Nevertheless, statements in the literature are quite vague, only mentioned as side notes (Bracke *et al.* 1978), and/or they originate from times, where the sterility of the cockroaches could only be checked via imprecise plating which lacks the power to detect unculturable microorganisms for example in the case of a study by Clayton (1959). There is only one detailed modern study showing a prolonged developmental time to the 5. instar of about 7.5 days in *P. americana* (Jahnes *et al.* 2019). Therefore, we used our established germ-free cockroach system to approve those studies and to gain further details especially on the real length of the delay in *B. germanica*.

Material and methods

Insects

A breeding culture of sequential *B. germanica* cohorts was established at the BAM in June 2015, initially obtained from the collection at the Federal Environment Agency, Berlin, which consists of a mixed population of 2 independent genetic backgrounds maintained for approximately 60 generations. Each generation consists of a minimum of 150 breeding pairs of cockroaches to minimize the effects of inbreeding. Each experimental cohort was maintained for approximately 190 days at 26 °C and 50 % humidity with a 12 h light-dark-cycle.

Ootheca collection and establishment of the germ-free cockroach breeding

To establish the first generation of germ-free *B. germanica* cockroaches, which are only inhabited by their essential symbiont *Blattabacterium sp.*, ootheca carrying females were anaesthetized with CO_2 and mature green line ootheca (Fig. 3.1) were collected directly from their abdomens. The use of mature ootheca is mandatory to prevent juveniles from drying out in the ootheca, since the green line only becomes clearly visible on the ootheca within 1 week before hatching which shortens the time without water supply from the mothers (Rust *et al.* 1995; Mullins *et al.* 2002).

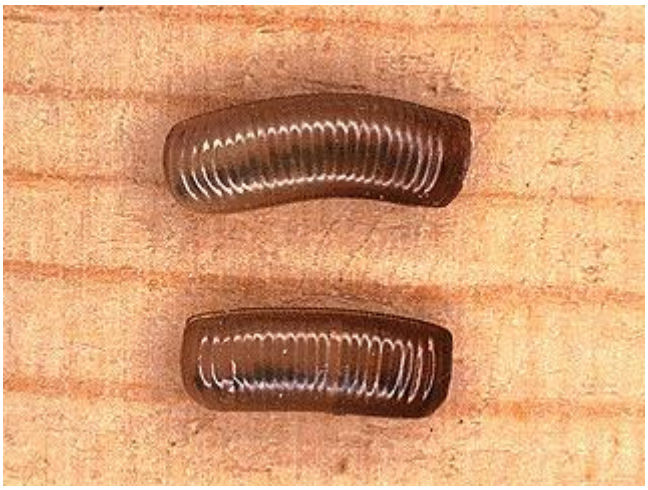

Fig. 3.1: Green line ootheca of the German cockroach *B. germanica* (taken from Pestchaser.com.au)

To surface-sterilize the ootheca a modified version of Tegtmeier's *et al.* (2016) protocol was used. All actions were performed in a clean bench under sterile conditions. The ootheca were first rinsed in 0.1 % sodium dodecylbenzenesulfonate and then transferred for 10 min to 3 % peracetic acid. After that they were rinsed in sterile water. Since this method led to poor results it was combined with another method by Benschoter and Wrenn (1972). In this case the ootheca were first rinsed in 0.1 % sodium dodecylbenzenesulfonate and then transferred for 10 min to 3 % peracetic acid. Another transfer followed for 10 min to 0.25 % sodium hypochlorite. In this case they were also rinsed in sterile water at the end.

The surface-sterilized ootheca were then placed in pairs in sterile 50 ml tubes, plugged with sterile cotton and the lids were loosely sealed. They were incubated at 26 °C and 75 % humidity till hatching. Thereafter, they were anaesthetized with CO_2 and transferred to sterile glass

bottles containing sterile cotton, food (for the exact composition see '**Chapter I** – **Materials and Methods**: **Insects and bacteria**') and 2 % agar (as a water source; both in 50 ml tubes; sterilized by autoclavation; Fig. 3.2). These breeding bottles were stored at 26 °C and 50 % humidity.

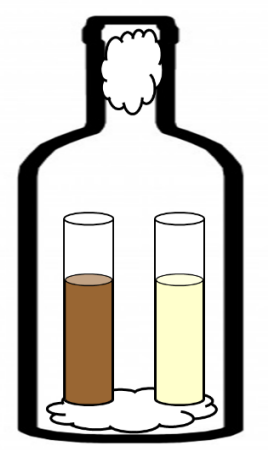

Fig. 3.2: Setup of a germ-free cockroach breeding bottle. There is autoclaved cotton on the bottom and as a plug. The 50 ml tubes contain either autoclaved food (brown) or autoclaved 2 % agar (light-yellow).

This procedure was further improved by an antibiotic treatment by adding 200 µg/ml rifampicin and 100 µg/ml gentamicin to the as a water source serving agar. These concentrations most likely do not harm the obligate endosymbiont *Blattabacterium sp*., since otherwise the cockroaches should not be able to develop into fertile adults under normal circumstances (Brooks and Richards 1955; Rosas *et al.* 2018). Nevertheless, the status of the *Blattabacterium sp.* still needs to be confirmed.

The subsequent generations of sterile cockroaches were established by transferring up to 2 ootheca carrying germ-free females to new, sterilized breeding bottles which were also stored at 26 °C and 50 % humidity.

Sterility testing by plating and 16S sequencing

All actions were performed in a clean bench under sterile conditions. The sterility of the germ-free cockroaches in a breeding bottle was tested in two ways. First, an individual from the

bottle to be tested was plated on LB-agar. The plated cockroach was incubated at 26 °C for 48 h. Second, from bottles successfully tested for the absence of microbial growth on plates up to 4 individuals (2 males 2 females) were used for 16S rDNA sequencing to additionally account for the presence of unculturable microbes. Together with them conventional cockroaches were also sequenced to examine the natural *B. germanica* microbiome. Their breeding was set up in the same way as it was for the germ-free cockroaches but without applying surface sterilization of ootheca or antibiotics. In total 40 conventional and 45 germ-free individuals from 14 and 15 flasks, respectively, were sequenced. Since cockroaches which already showed no microbial growth on plates were also tested sufficiently sterile using 16S rDNA sequencing, we decided to test the sterility of the germ-free cockroaches which were produced by ootheca surface sterilization in combination with serving antibiotics in the agar only by plating. Microbial DNA was isolated using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer with some modifications. Briefly, the cockroaches were surface-sterilized prior to the extraction by soaking in 70 % ethanol, followed by a transfer to 10 % sodium hypochlorite for 1 min and then a rinse in sterile water (Hammer *et al.* 2015). A piece of a sterile pipet tip was treated in the same way to also include the surface sterilization process as a negative control (negative tip control). This negative control is further treated in the same way as the cockroach sample, just the following homogenization step is skipped. After sterilising its surface, each cockroach was homogenized twice at 4 m/s for 15 s in a homogenizer (FastPrep™-24, MP Biomedicals) in 120 µl C1 solution by using a 5 mm steel bead (Qiagen) and then 60 µl were transferred into the PowerBead tube together with 100 µg Proteinase K and incubated over night at 56 °C. During this step another negative control (negative control) was introduced where instead of the cockroach nothing was introduced to account for potential microbial DNA contaminants in the kits being used. This control was further treated in the same way as the real samples. The initial homogenization procedure was necessary since the beads supplied by the manufacturer were not strong enough to break the cockroach cuticle. The remaining 60 µl could be stored at -80 °C for potential repetitions. The extractions were completed using the entire PowerSoil protocol and all samples were eluted in 50 µl of solution C6 (Rubin *et al.* 2014).

PCRs on the isolated DNA samples were performed using the KAPA2G Fast HotStart PCR ready mix (Kapa Biosystems) and the 515f_1n_fus (TCGTCGGCAGCGTCAGAT GTGTATAAGAGACAGNGTGYCAGCMGCCGCGGTAA) and 806r_1n_fus (GTCTCGTGG GCTCGGAGATGTGTATAAGAGACAGNGGACTACNVGGGTWTCTAAT) adapter primers from the earth microbiome project (Thompson *et al.* 2017).

The exact PCR composition was as follows:

- 12.5 µl KAPA2G Fast HotStart PCR ready mix
- 1.25 µl 515f_1n_fus forward primer
- 1.25 µl 806r_1n_fus reverse primer
- 5.0 µl isolated DNA template (around 50 ng)
- 5.0 µl nuclease-free water.

The PCR conditions were as follows:

1. 95 °C: 3 min
2. 95 °C: 15 s
3. 60 °C: 15 s
4. 72 °C: 15 s
5. 72 °C: 3 min

Steps 2 – 4 were repeated for 30 cycles. Afterwards the PCR products were visualized on a 1.5 % Tris-acetate-EDTA-agarose-gel.

The PCR products were then used for the library preparations for the 16S rDNA sequencing according to the BeGenDiv protocol (Appendix) starting with step 2.

Sequence quality control and taxonomic analyses

Sequencing reads were trimmed, denoised and overlapped using a full-stack R pipeline (Callahan *et al.* 2016a; R 3.6.0 (R Core Team 2019)) incorporating dada2 v1.12.1 (Callahan *et al.* 2016b) and phyloseq v1.28.0 (McMurdie and Holmes 2013). Forward and reverse reads were trimmed to 200 bp, truncated at the first instance of a quality score less than 2 and filtered on a maximum expected error rate of 2 errors per truncated read. The remaining forward and reverse reads were dereplicated and denoised using a parameterized model of substitution errors. The resulting denoised read pairs were merged and subjected to *de novo* chimera removal. Taxonomy was assigned using the Ribosomal Database Project training set v16. The resulting exact sequence variants were agglomerated at the genus level. Shannon indices, beta diversity distance matrices, and ordinations were calculated using phyloseq v1.28.0 (McMurdie and Holmes 2013).

Differences in Shannon indices among the treatments were estimated using a linear model. Comparisons among treatment levels were carried out with post-hoc Tukey tests using a Bonferroni correction, using package multcomp v1.4-10 (Hothorn *et al.* 2008).

Whether the beta diversity (Bray-Curtis index) of the treatments differ significantly among each other was further tested by converting the abundances to relative abundances followed by permutational analysis of variance using the adonis function of vegan v2.5-6 (Oksanen *et al.* 2019). *P*-values of multiple comparisons were Bonferroni-corrected.

Comparison of the developmental time of conventional and germ-free cockroaches

The developmental time of conventional and germ-free cockroaches was compared by using 19 replicates (flasks) per treatment. The conventional cockroaches were set up in the same way as the germ-free ones but without performing the surface sterilization and the use of antibiotics. Only second-generation individuals were used. All replicates were checked daily and the date of hatching and the date of molting into adults (the day when both sexes were present) were recorded. The timespan in days between those two events was defined as the developmental time.

The developmental times of conventional and germ-free *B. germanica* cockroaches were analysed using a Wilcoxon rank sum test in R 3.6.1 (R Core Team 2019).

Results

Sterility testing by plating and 16S sequencing

Plating on LB-agar revealed that treating *B. germanica* ootheca only with 0.1 % sodium dodecylbenzenesulfonate and 3 % peracetic or additionally with 0.25 % sodium hypochlorite led to cockroaches free of culturable microbes in 40 % of all cases. In most cases a rudimentary microbiota was still present (example plate is shown in Fig. 3.3). Raising cockroaches on the antibiotics rifampicin (200 µg/ml) and gentamicin (100 µg/ml) improved the absence of culturable microorganisms to 99 % (example plate is shown in Fig. 3.3).

Fig. 3.3: Example pictures of *B. germanica* cockroaches plated on LB-agar. **Left**: conventional cockroach; **Middle**: semi-germ-free cockroach hatched from an only surface-sterilized ootheca; **Right**: true germ-free cockroach hatched from a surface-sterilized ootheca and being raised on antibiotics. A true germ-free cockroach hatched from an only surface-sterilized ootheca shows the same pattern.

The 16S rDNA gene of potential microbes from cockroaches either sterilised only with peracetic acid or with peracetic acid in conjunction with bleach which featured no microbial growth on LB-plates was also sequenced to further investigate abundance of unculturable microbes. In concert with them conventional control cockroaches and negative controls (negative and negative tip control) were sequenced as well to have a more precise estimation of the effectiveness of the sterilization methods and to gain further in sights up on the microbiota of conventional cockroaches. The Shannon index (Fig. 3.4) of the conventional *B. germanica* individuals was the highest of all treatments. The negative control and the negative tip control showed intermediate indices, while being not significantly different from each other (negative control vs. negative tip control: t = -0.204, p = 1.000). The Shannon index of germ-free cockroaches was the lowest (Fig. 3.4A) and the two different sterilization methods (acid; acid + bleach; Fig. 3.4B) showed no difference among each other (germ-free acid vs. germ-free acid + bleach: t = -0.911, p = 1.000). Therefore, the two sterilization methods were summarized as germ-free and the two negative controls as negative control for subsequent analysis which showed that they differ significantly from the conventional ones regarding their Shannon indices (germ-free vs. conventional: t = -15.334, p < 0.001). The Shannon indices of both, conventional and germ-free cockroaches were each significantly different from the negative control (germ-free vs. negative control: t = 7.803 , p < 0.001; conventional vs. negative control: t = -2.457, p = 0.049).

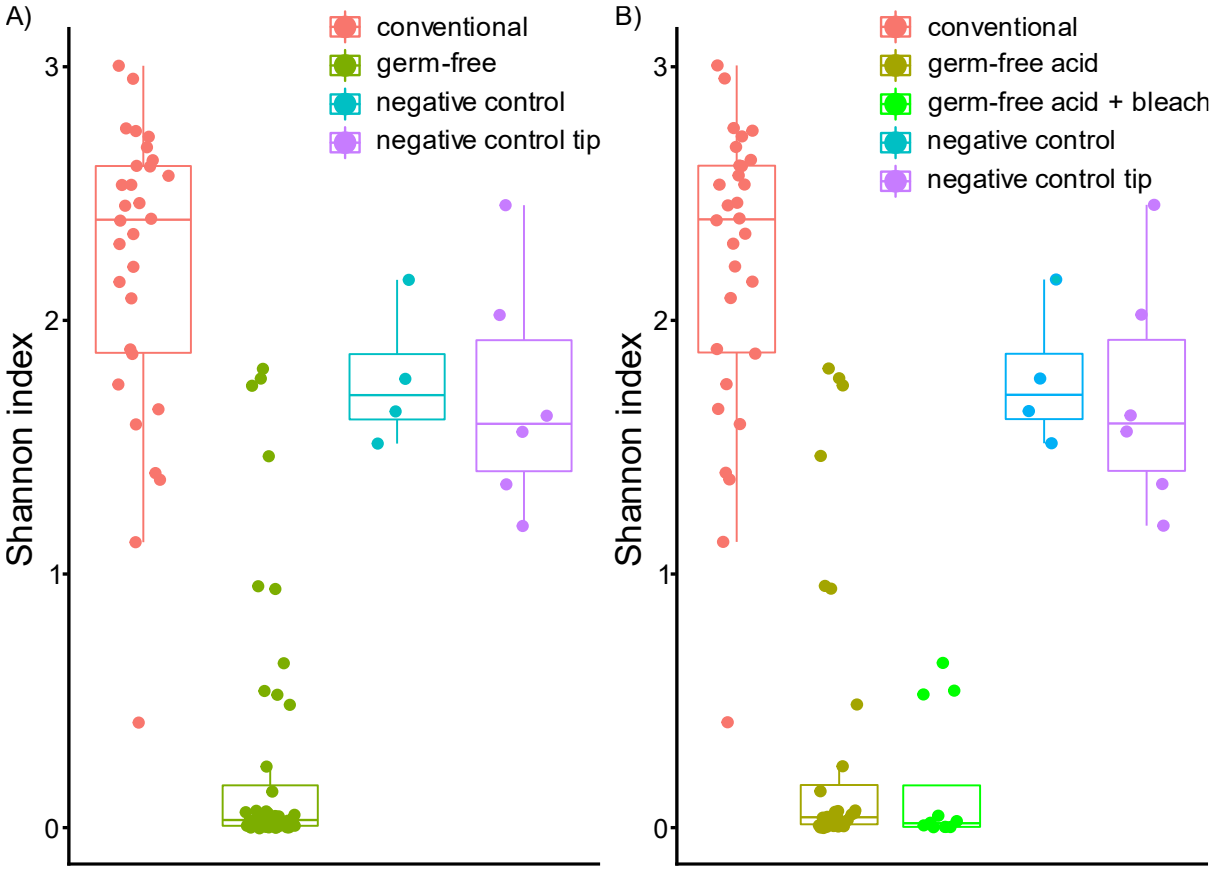

Fig. 3.4: Shannon indices of **A)** conventional and germ-free *B. germanica* cockroaches **B)** conventional and germ-free *B. germanica* cockroaches according to the sterilization technique used. Negative control accounts for the chemicals used for the DNA isolation and the library preparation and negative control tip accounts for the surface sterilization of individuals right before sequencing (see '**Sterility testing by plating and 16S sequencing**'). 16S sequencing was used to do these comparisons. Dots represent an individual of the given treatment.

Analyses of the β-diversity of the bacterial communities showed similar results with groups being separated by treatment (Fig. 3.5A). Both the two different sterilization methods for the germ-free ones and the two control groups showed no difference among each other (Fig. 3.5B).

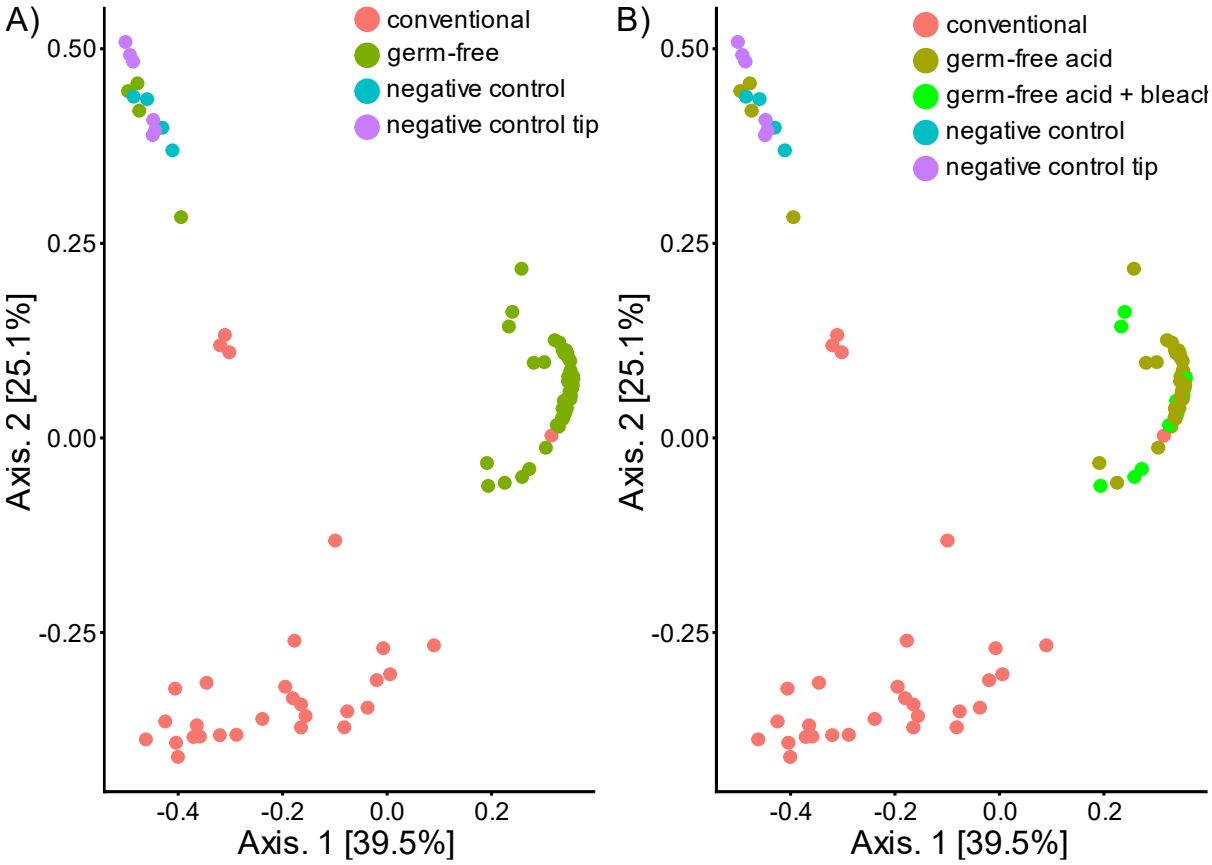

Fig. 3.5: β-diversity of bacterial communities found in **A)** conventional and germ-free *B. germanica* cockroaches **B)** conventional and germ-free *B. germanica* cockroaches according to the sterilization technique used. Negative control accounts for the chemicals used for the DNA isolation and the library preparation and negative control tip accounts for the surface sterilization of individuals right before sequencing (see '**Sterility testing by plating and 16S sequencing**'). 16S sequencing was used to do these comparisons. Dots represent replicates.

Cockroaches from the different sterilization methods did not differ significantly from each other in their β-diversity (PERMANOVA: F = 1.2499, $p > 0.1$), similarly for the two negative control groups (PERMANOVA: F = 0.50138, $p > 0.1$). Therefore, the two sterilization methods were summarized as germ-free and both negative controls as negative control for subsequent analyses. In most cases germ-free cockroaches featured no or only rudimentary microbial signatures significantly differing from conventional ones regarding the β-diversity (PERMANOVA: F = 65.693, $p = 0.003$). The β-diversity of both, conventional and germ-free cockroaches were each significantly different from the negative control (PERMANOVA: germ-free vs. negative control: F = 74.986, $p = 0.003$; conventional vs. negative control: F = 40.401, $p = 0.003$).

In *B. germanica* cockroaches the taxonomic composition can be well described by the 10 most abundant genera (Fig. 3.6) which are *Alistipes*, *Anaerotruncus*, *Bacteroides*, *Desulfovibrio*, *Dysgonomonas*, *Intestinimonas*, *Lactococcus*, *Parabacteroides*, *Serratia* and *Xenorhabdus* (further taxonomic information is given in Tab. 3.1). On average each sample contained 7.2 of these genera with a range from 3 to 10 genera represented. The endosymbiont *Blattabacterium sp.* is contained within the group of no taxonomic designation (other) in all cockroach samples. In the cases regarding the germ-free cockroaches where rudimentary microbiota beyond the *Blattabacterium sp.* (2 most abundant sequences) was present those microorganisms belong to the genera *Alistipes*, *Bacteroides*, *Desulfovibrio*, *Dysgonomonas*, *Intestinimonas*, *Lactococcus*, *Parabacteroides*, *Serratia* or *Xenorhabdus* with the genus *Anaerotruncus* being undetectable in germ-free *B. germanica* individuals (Fig. 3.6). On average each germ-free sample cockroach contained 1.3 of these genera with a range from 0 to 5 genera being represented and 14 samples containing 0 genera. The abundances of those genera were also quite distinct from the ones of the conventional cockroaches and the microbial genera found in the negative controls are completely different from the ones found in the cockroaches (Fig. 3.6).

Tab. 3.1: Taxonomy of the 10 most abundant genera in conventional *B. germanica*. Taxonomic information is taken from the NCBI taxonomy (Benson *et al.* 2009; Sayers *et al.* 2009).

Genus	Family	Phylum
Alistipes	Rikenellaceae	Bacteroidetes
Bacteroides	Bacteroidaceae	
Dysgonomonas	Dysgonamonadaceae	
Parabacteroides	Tannerellaceae	
Anaerotruncus	Ruminococcaceae	Firmicutes
Intestinimonas	unclassified Clostridiales	
Lactococcus	Streptococcaceae	
Desulfovibrio	Desulfovibrionaceae	Proteobacteria
Serratia	Yersiniaceae (Gammaproteobacteria)	
Xenorhabdus	Morganellaceae (Gammaproteobacteria)	

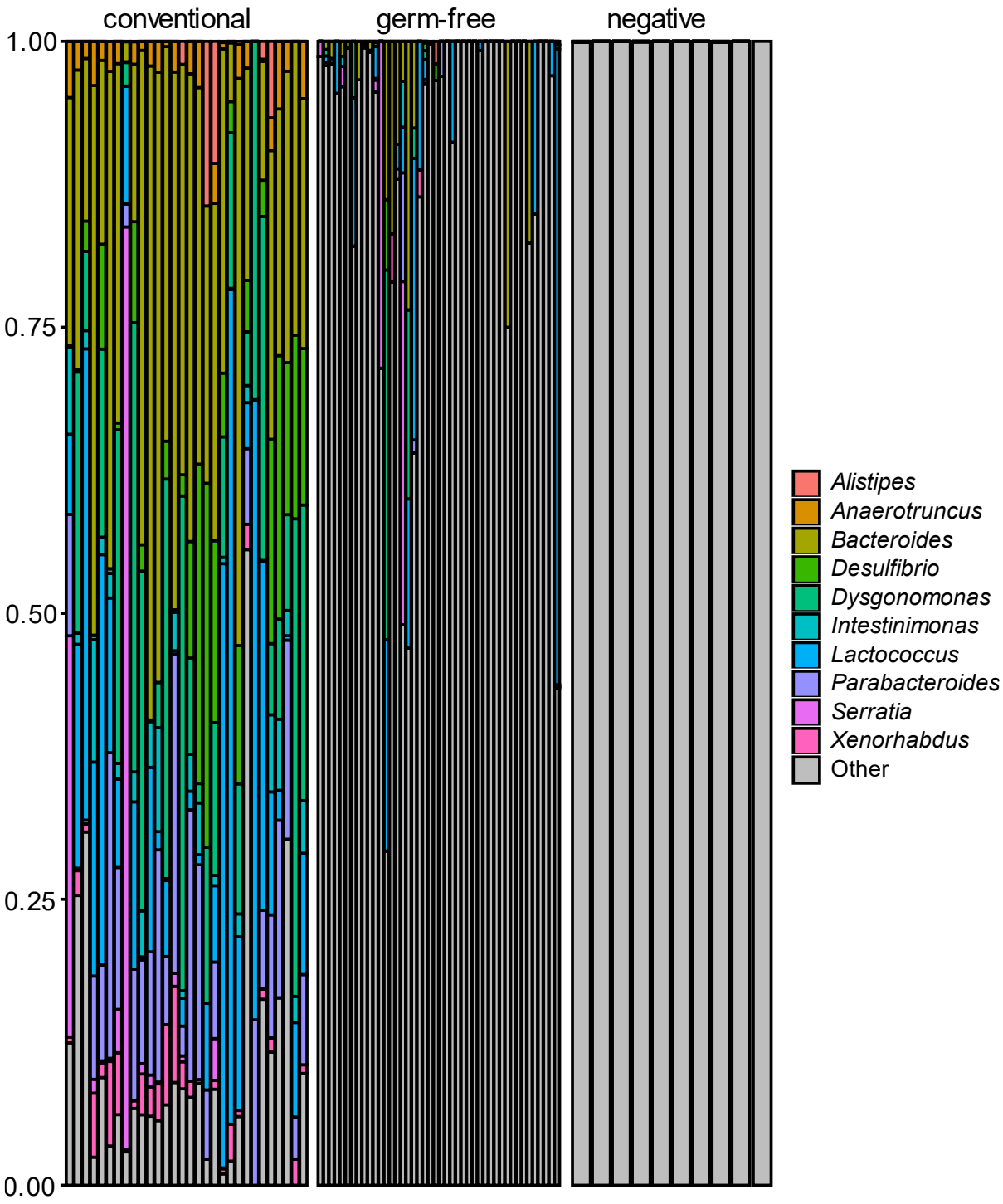

Fig. 3.6: Abundance of the 10 most common genera of *B. germanica* cockroaches in conventional and germ-free cockroaches as well as in the negative controls. The obligate symbiont *Blattabacterium sp.* is represented within the genera 'other' and each column represents an individual cockroach or a negative control sample.

Comparison of the developmental time of conventional and germ-free cockroaches

Germ-free *B. germanica* cockroaches (mean developmental time both sexes present: 77.5 days) needed approximately 35 days longer from the day of hatching to the day when they turned into adults than their conventional counterparts (mean developmental time both sexes present: 42.9 days; Wilcoxon rank sum test: W= 10.0, $p < 0.001$; Fig. 3.7). Additionally, they featured a greater variance. They molted into adults within a 61-day time frame whereas the conventional ones molted within a 6-day time frame.

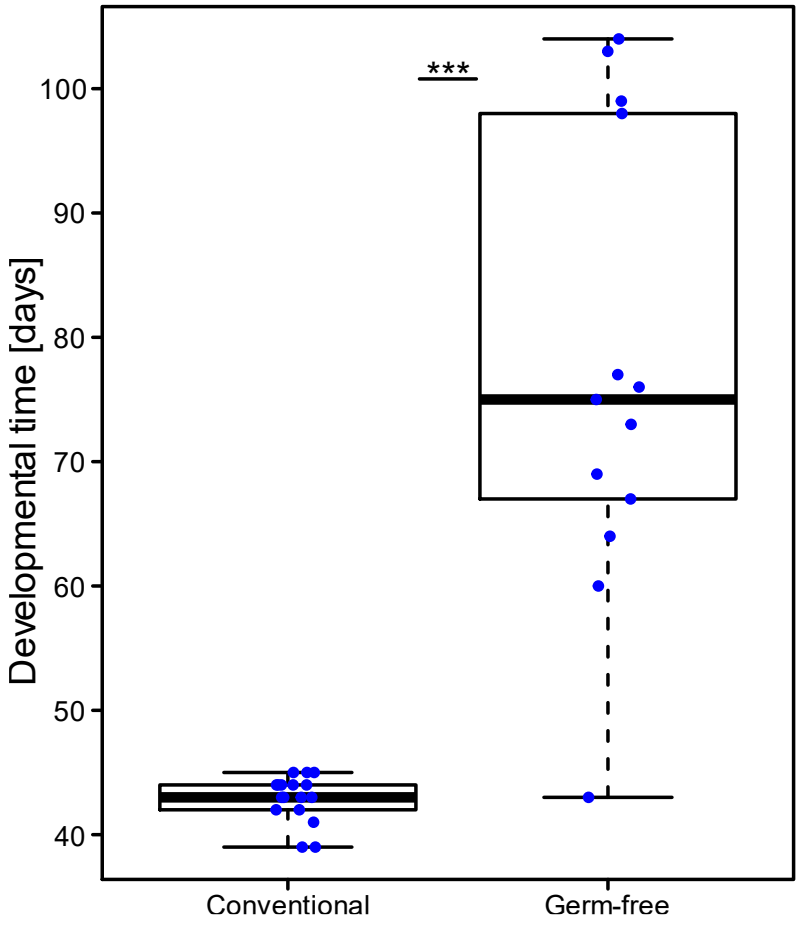

Fig. 3.7: Developmental time of conventional and germ-free cockroaches in days. Each blue dot represents a different breeding batch. The median is indicated by the bold black line within the box plot.

Discussion

Our study reveals that although sterilization techniques for the generation of germ-free cockroaches are already known for several decades there is still room to further improve them. In the beginning we only used a wash in 3 % peracetic for 10 min to surface-sterilize mature *B. germanica* ootheca. According to Doll *et al.* (1963) using 0.1 % peracetic acid for 10 min resulted in *B. germanica* cockroach cultures with no demonstrable bacteria when food and faeces were tested. This result is already at least somewhat outdated by a study on another cockroach species. Tegtmeier *et al.* (2016) showed that sterilization of *S. lateralis* ootheca was not reliable at acid concentrations below 0.5 %. They report that 2 % peracetic acid for 5 min results in germ-free juvenile cockroaches in 98 % of the cases. Contrary to these two studies the use of peracetic acid alone even at 3 % for 10 min was not enough in our case to result in germ-free adult cockroaches in more than 40 % of the cases as examined by plating assays. This result was also not further improved when we added a subsequent sterilization step using 0.25 % sodium hypochlorite for 10 min, a chemical which was also used by Benschoter and Wrenn (1972) to create germ-free *B. germanica* cockroaches. They used 0.25 % sodium hypochlorite for 20 min and encountered bacterial contaminations in only 2 cases within a year. Notably, similar to Doll *et al.* (1963) they also only tested the sterility of faeces, bits of diet as well as their equipment but never adult cockroaches. All these findings indicate that the applied sterility testing method as well as the time point of testing seem to be very critical for the outcome and therefore their evaluation. There is the chance that microbial contaminants might be missed if only the surrounding and not the cockroach itself is examined or if they are examined in early life stages when rudimentary microbes which survived the sterilization probably had not enough time to re-establish in detectable abundances. Furthermore, there seems to be a species effect as well since in *S. lateralis* surface sterilizations with peracetic acid even with modern verification methods was sufficient (Tegtmeier *et al.* 2016). The difference here is quite likely the nature of the surface of the ootheca. For *S. laterails* (Fig. 3.8) it is smooth in most parts while for *B. germancia* (Fig. 3.1) it is covered with grooves in which at least some microbes might survive the surface sterilization treatment. In our case only a treatment with antibiotics following the surface sterilization led to germ-free adult *B. germanica* cockroaches in 99 % of all cases when sterility was checked by plating.

Fig. 3.8: Ootheca of the cockroach species *S. lateralis* (taken from Tegtmeier *et al.* 2016).

Nevertheless, cockroach breeding batches created only by surface sterilization without the use of antibiotics which were tested germ-free according to the plating protocol are confirmed to harbour only the unculturable *Blattabacterium sp*. or a rudimentary microbiota.

The microbiota of conventional *B. germanica* males was dominated by Bacteroidets, Firmicutes and Proteobacteria (Tab. 3.1) which were also identified by others as dominant phyla due to the omnivorous nature of cockroaches (Rosas *et al.* 2018). This microbiota can be further well described by 10 most common genera (Fig. 3.6; Tab. 3.1) which were present beside the unclassified ones which contained the *Blattabacterium sp*.. These genera were *Alistipes*, *Anaerotruncus*, *Bacteroides*, *Desulfovibrio*, *Dysgonomonas*, *Intestinimonas*, *Lactococcus*, *Parabacteroides*, *Serratia* and *Xenorhabdus* with conventional cockroaches harbouring on average 7.2 of those. In contrast, germ-free *B. germanica* males harboured on average only 1.3 genera (in addition to the *Blattabacterium sp.*) including *Alistipes*, *Bacteroides*, *Desulfovibrio*, *Dysgonomonas*, *Intestinimonas*, *Lactococcus*, *Parabacteroides*, *Serratia* or *Xenorhabdus* with *Anaerotruncus* always being absent (Fig. 3.6). The families which correspond to the genera we found in conventional *B. germanica* males (Tab. 3.1) are in large parts in concordance with a study by Pérez-Cobas *et al.* (2015) who examined the microbiota of *B. germanica* as well. Just like us they found the families Rikenellaceae, Bacteroidaceae (phylum Bacteroidetes), Ruminococcaceae, Desulfovibrionaceae (phylum Proteobacteria) and unclassified Clostridiales (phylum Firmicutes) with the first 4 belonging to the core microbiota of *B. germanica* as classified by the authors (Pérez-Cobas *et al.* 2015). In contrast, the families Dysgonamonadaceae, Tannerellaceae (phylum Bacteroidetes), Streptococcaceae (phylum Firmicutes), Yersiniaceae and Morganellaceae (phylum Proteobacteria) are absent in their study. This could be local characteristics since the composition of the microbial community is strongly influenced by environmental factors especially the diet (Sabree *et al.* 2012; Pérez-Cobas *et al.* 2015).

The differences between all treatments are further corroborated by differing Shannon indices (Fig. 3.4). The conventional individuals featured the highest indices while the germ-free ones featured the lowest. Both negative controls which account for microbial contaminations during the DNA extraction and the library preparation and the negative tip controls which account for the pickup of microbial contaminants during the surface sterilization of adult cockroaches right before the DNA extraction showed intermediate Shannon indices (Fig. 3.4). This suggests that the laboratory equipment or the DNA extraction kit used were contaminated with microbial DNA. As indicated by several studies, DNA extraction kits and laboratory reagents are a considerable source of microbial contamination in microbiome studies (Salter *et al.* 2014; Weiss *et al.* 2014; Glassing *et al.* 2016). This issue becomes particularly problematic for low biomass samples because as the 'true' target sequences become smaller, the potential for contaminants occupying a larger fraction of the sequences will become greater (Salter *et al.* 2014; Weiss *et al.* 2014; Glassing *et al.* 2016). Here the 'small biomass' problem also explains the intermediate Shannon indices of both negative controls. Those samples should inherently contain no biomass at all whereas samples from germ-free cockroaches still contain the biomass of *Blattabacterium sp*. in large amounts. Therefore, contaminations were more frequently picked up in the negative controls than in the germ-free ones resulting in higher microbial diversity levels and a more even abundance of those microbial traces as represented by Shannon indices. Nevertheless, traces of microbes found in both negative controls are very distinct from the ones found in conventional or germ-free cockroaches (Fig. 3.6). Shannon indices were the greatest for conventional cockroaches with average Shannon indices of 2.4 (Fig. 3.4). These results were further supported by analyses regarding the β-diversity where all major treatment groups were separated with only the two different sterilization methods and the two sterilization methods overlapping (Fig. 3.5).

Cockroaches which were shown to be sterile or which featured just rudimentary microbiota were also examined in a study regarding their developmental time. Germ-free *B. germanica* cockroaches needed on average 35 days longer to develop into adults than their conventional counterparts harbouring an intact microbiota (Fig. 3.6). A finding which is further supported by a study on the same species which could also show a longer maturation time for germ-free individuals (Clayton 1959) and two other studies on different cockroach species. Juveniles of the cockroach *P. americana* grow and develop slower when their gut microbiota is cleared through antibiotic treatment (Bracke *et al.* 1978) or ootheca surface sterilization (Jahnes *et al.* 2019). Reasons for the impact of microbial life on host development seem to be due its impacts on host nutrition. Storelli *et al.* (2011) for instance could show that reintroducing the commensal microbiota into germ-free *D. melanogaster* larvae raised on a poor medium is sufficient to facilitate developmental growth and accelerate developmental timing. Genetic data

furthermore indicates the role of nutrition by showing that the microbiota activates the fly's nutrient sensing system, thus leading to enhanced systemic hormonal growth signaling and faster development (Storelli *et al.* 2011).

In conclusion, we could show that the way of determining the success of methods to clear microbiota is particularly important since it can drastically influence the outcome. In our case surface sterilization was not enough to create germ-free *B. germanica* individuals which is contradictory to other studies which however mostly only used indirect testing of food and equipment or testing of hatchlings too early in time. Only the combination of surface sterilization with peracetic acid and sodium hypochlorite followed by an antibiotic treatment with rifampicin and gentamicin was enough to create cockroaches only inhabited by their mandatory *Blattabacterium sp.* symbiont together with a very rudimentary microbiota as indicated by direct plating and 16S sequencing of adults. Furthermore, we could show that germ-free *B. germanica* cockroaches needed significantly longer to develop into adult individuals. Combined with the fact, that microbes not only support development in invertebrates, but also in vertebrates where they are for example required in both fish and mammals for gut development, underlines that these host-microbe interactions are very successful and therefore highly conserved (Fraune and Bosch 2010).

Chapter III

Be clean, be healthy? Impact of the microbiome on immune gene regulation and the outcome of a bacterial infection in the cockroach *Blatella germanica*

Abstract

1. Million years of coevolution led to manifold interactions between the physiologies of the microbiome and their hosts including developmental, nutritional and immunological functions. Studying these relationships in humans and other vertebrates is extremely difficult because the complexity of their microbial composition is quite high. Most of these microbes are extremely hard to culture and raising these animals in a strictly sterile environment is very cost intensive. In insects, however, it is much easier to perform such studies and these studies can parallel to what is happening in humans or other animals. Insects also have quite simple microbial communities with many of its members being easily cultivatable and the insect hosts can be kept under sterile conditions with small efforts.
2. In the first part of this study, we performed transcriptomic studies on conventional and germ-free male *B. germanica* cockroaches using two different published genomes (either Harrison *et al.* 2018 or He 2018) for gene identification to gain insights on the microbial impacts on host gene expression.
3. In the second part, we further combined this with the examination of the impact of the microbiome on immune parameters by following the survival of germ-free and conventional cockroaches after infection with the opportunistic insect pathogen *P. entomophila*.
4. We found that that there were differences in the numbers of genes being identified and differentially expressed between the two reference genomes being used. From the genome annotation by Harrison *et al.* (2018) 25451 putative genes were identified and 184 of those, including 19 immune-related genes, were significantly different expressed between conventional and germ-free cockroaches. Form the genome annotation by He (2018), 111778 putative genes were identified and 1082 of those, including 30 immune-related genes, were significantly different expressed between conventional and germ-free cockroaches. Together with that conventional *B. germanica* males died faster and displayed a higher mortality after *P. entomophila* infection than their conventional counterparts.
5. We showed that beside differences on the results due to different reference genomes being used for transcriptomic studies comparing germ-free and conventional *B. germancia* males, the take-home message is that the presence of an intact microbiota heavily influences the expression of several host genes including immune-related ones. This suggests that the latter assists the cockroach in combating infections as

expressed by the survival data. These findings altogether highlight that the microbiome is not only impacting one but all parts of the host’s biology.

Keywords: transcriptome, immunity, germ-free, microbiome

Introduction

The ‘hologenome theory’ by Zilber-Rosenberg and Rosenberg (2008) is based on four generalizations: 1) all animals and plants establish symbiotic relationships with diverse microorganisms; 2) symbiotic microorganisms can be transmitted between generations with fidelity; 3) the association between host organism and its microbial community affects the fitness of the holobiont within its environment; 4) genetic variation in holobionts can be enhanced by incorporating different symbiont populations and can change under environmental demand more rapidly and by more processes than the genetic information encoded by the host organism alone.

It is quite likely that, at least for the vertebrate gut microbiota, this relationship mainly evolved for nutritional purposes (Ley *et al.* 2008; reviewed in the general introduction). This is already indicated by the interactions of different groups of bacteria in biofilms and microbial mats with the exchange of metabolites (Rosenberg *et al.* 2010) and further pronounced by the various mutualistic symbiosis of animals and their microbiota which involves the degradation of challenging dietary compounds (Fraune and Bosch 2010; Karasov *et al.* 2011). However, million years of coevolution have led to other pervasive interactions between the physiologies of the microbiome and the host which reach beyond nutritional functions (Hooper *et al.* 2012). These interactions are particularly apparent when host immune function is concerned (Hooper *et al.* 2012).

Studying these relationships presents a considerable impediment in humans and other vertebrates, because of the high complexity of the microbial composition, the difficulty to culture most of these microbes, and the cost of raising these animals in a strictly sterile environment (Ma *et al.* 2015). In contrast, insects represent a great system to perform such studies because they display far greater diversity than mammals in their interactions with microbes, with some remarkable associations concerning morphological intimacy and molecular integration (Douglas 2011). In addition, they also have rather simple microbiomes with many of its microbial members being easily cultivatable and their hosts being raiseable under sterile conditions with low efforts (Ma *et al.* 2015).

Firstly, it is notable, that the immune system plays a critical role in the persistence of the microbiome. The insect immune system includes both cellular and humoral immunity but lacks adaptive immunity (Lavine and Strand 2002). The cellular immunity comprises hemocyte-mediated phagocytosis, encapsulation and nodulation (Lavine and Strand 2002). The humoral immunity comprises three main immune pathways: the Toll pathway, the Imd pathway and the

Jak/Stat pathway (Hillyer 2016). They are activated through the recognition of microbial structures by proteins called pattern recognition receptors (PRRs) which are targeting structures like bacterial peptidoglycan, lps, lipoteichoic acids and fungal glucans (reviewed in Feldhaar and Gross 2008). PRRs which recognise bacterial peptidoglycan are called peptidoglycan recognition proteins (PGRPs) while the ones which bind bacterial lps, lipoteichoic acids or fungal glucans are called Gram-negative binding proteins (GNBPs; reviewed in Feldhaar and Gross 2008). The main feature of the immune pathways is the production of AMPs, but they also consist of coagulation and melanisation cascades, and the production of reactive intermediates of oxygen and nitrogen (reviewed in Lavine and Strand 2002). AMPs are small peptides and proteins with antimicrobial properties produced by the fat-body and hemocytes (reviewed in Rosales 2017; Yakovlev *et al.* 2017).

In *D. melanogaster*, the gut epithelial cells have receptors for the Imd pathway and the resident gut microbiota can also trigger this pathway which potentially could lead to the production of AMPs. This stimulation does not occur though (Lemaitre and Hoffmann 2007; Ryu *et al.* 2008). The reason is the gut specific homeobox gene Caudal which regulates the commensal-gut mutualism by repressing nuclear factor kappa B–dependent AMP genes (Ryu *et al.* 2008) and there are additional negative regulators which sequester PGRP-LC in the cytoplasm and thereby also prevent the activation of the Imd pathway (Kleino *et al.* 2008; Lhocine *et al.* 2008). When Caudal expression is reduced by double-stranded RNA-mediated interference (RNAi), a process by which introduced double-stranded RNA is degenerated into short RNAs that activate ribonucleases to target homologous mRNA (Agrawal *et al.* 2003), AMP gene expression is upregulated. Importantly, this does not lead to the complete elimination of the gut microbiota, but instead it leads to a change in the microbial composition (Ryu *et al.* 2008). This implies, that AMPs are not exclusively antagonistic to microorganisms, but can also act to manage and regulate the microbial community (Douglas 2011) and this fact is also supported by another study on the mosquito species *A. aegypti* and *Culex pipiens pallens* (Pang *et al.* 2016). Further evidence that the innate immune system shapes coexistence of insects and their microbiota comes from studies on intracellular bacteria in *Sitophilus* (weevils; Vigneron *et al.* 2012) and *Glossina* (tsetse flies; Wang *et al.* 2009) where PGRP-LB is highly expressed in the bacteriocytes. This expression leads to the removal of the peptidoglycan ligand that triggers the Imd pathway.

However, the microbiota can in return also greatly affect host health by modulating the host's immune system. A study on tsetse flies could show that an intact microbiota is needed to establish a full functioning immune system, since the absence of an obligate symbiont led to the development of adult flies exhibiting a compromised immune system (Weiss *et al.* 2012). Another study on tsetse flies and fruit flies could additionally show that an intact microbiota is

already required in the larvae to express a functioning melanisation response in adult stages (Benoit *et al.* 2017). In *A. aegypti* mosquitos the microbiota is needed for the activation of the Imd pathway in the face of a *Sindbis* virus infection (Barletta *et al.* 2017). Furthermore, several studies could show that the host microbiota can also directly combat parasite and pathogen infections (reviewed in the general introduction).

Beside all this, not much is known about the influence of the microbiota on the overall gene regulation in the host and especially on immune gene expression outside of some prominent model insect species. There are a few studies on *D. melanogaster,* the *Anopheles* complex, honeybees and tsetse flies showing that the presence of the microbiota significantly alters the expression of a core set of genes that control transcription, gut structure, metabolism, signalling, stress response and last but not least immunity (Xi *et al.* 2008; Dong *et al.* 2009; Wang *et al.* 2009; Broderick *et al.* 2014; Combe *et al.* 2014; Guo *et al.* 2014; Kwong *et al.* 2017). Cockroaches are especially suited to gain further insights, since they can be reared under germ-free conditions (see **Chapter II**) and they feature effective strategies to combat pathogens since they are frequently exposed to a rich antigenic environment due to their lifestyle (Mayer *et al.* 2016). This is further supported by the published genomes of *B. germanica* (Harrison *et al.* 2018) and *P. americana* (Li *et al.* 2018) revealing the expansion of specific immune gene families. Therefore, we sequenced and compared the transcriptome of conventional and germ-free *B. germanica* cockroaches focusing especially on immune-related genes. To identify those genes the transcriptome was either aligned to the reference genome of Harrison *et al.* (2018) or to a *de novo* assembly by He (2018) and the resulting differences are discussed. We further supported our findings with *in vivo* survival experiments upon *P. entomophila* systemic infections.

Material and methods

Insect culture and germ-free cockroach production

B. germanica stock cultures were kept in the same way as reported in '**Chapter II – Material and methods: Insects'**. For the survival experiments, first generation conventional and germ-free cockroaches were produced according to '**Chapter II – Material and methods: Ootheca collection and establishment of the germ-free cockroach breeding'** using 0.1 % sodium dodecylbenzenesulfonate, 3 % peracetic acid and 0.25 % sodium hypochlorite, each for 10 min to surface-sterilize collected green-line ootheca. For the *de novo* transcriptome sequencing, second generation conventional and germ-free cockroaches were produced

according to '**Chapter II – Material and methods: Ootheca collection and establishment of the germ-free cockroach breeding'** using 0.1 % sodium dodecylbenzenesulfonate, 3 % peracetic acid and 0.25 % sodium hypochlorite to surface-sterilize collected green-line ootheca in combination with 200 µg/ml rifampicin and 100 µg/ml gentamicin supplied in the agar which served as a water source. The methods slightly differ because during the course of the experiments it became obvious that creating cockroaches using antibiotics leads to higher amounts of successful germ-free cockroaches. The cockroaches were kept at all time at 26 °C and 50 % humidity. All experiments were conducted with males to minimise changes in physiology associated with oogenesis.

Sterility testing by plating and 16S sequencing

All actions were performed in a clean bench under sterile conditions. For the survival experiments, we followed the protocol in '**Chapter II – Material and methods: Sterility testing by plating and 16S sequencing'.** Since cockroaches, which already showed no growth on plates, were also tested sterile using 16S sequencing, we decided to only test the sterility for the transcriptomic experiment by plating.

RNA isolation and purification

Total RNA was isolated from 9 whole conventional and from 9 whole germ-free male cockroaches from 3 different cohorts (3 individuals per cohort). Each cockroach was suspended in Trizol (Thermo Fisher Scientific), and homogenized with two 3 mm steel beads (Retsch) using a homogenizer (Retsch Mill MM300, Retsch) at maximum speed for 5 min. Recovery of total RNA was done by chloroform extraction and then cleaning up 200 µl of the aqueous phase using the RNAeasy Mini kit (Qiagen) starting with step 1 of the RNA clean up protocol according to the manufacturer's instructions. Minor changes to the original protocol were that the aqueous phase was first mixed with 700 µl Buffer RLT and 500 µl pure ethanol, and step 3 was done twice due to excess sample volume. Afterwards the optional DNAse digestion step was performed. The purified RNA was suspended in 50 µl RNAse-free water. Quantity and quality of the isolated RNA samples were determined by using the Agilent 4200 TapeStation system. In the rare case in which the RNA quality was not pure, the clean-up was performed again, but without any modifications to the manufacturer's instructions and without the DNAse digestion step.

De novo transcriptome sequencing

The library was prepared using NEXTflexTM Rapid Directional mRNA-seq kit (Bioo Scientific) according to manufacturer's instructions. Briefly, polyadenylated mRNA was enriched by poly-A beads out of 1 µg total RNA per sample. From each sample, first-strand and second-strand cDNA was synthesized, fragmented and barcoded with NEXTflexTM RNA-seq Barcode Adapters. The prepared library was sequenced on an Illumina NextSeq500/550 platform at the BeGenDiv according to their guidelines. Quantity and quality of the prepared library was determined by using the Qubit 2.0 Fluorometer (Thermo Fisher) and the Agilent 4200 TapeStation system.

Transcript abundance estimation and differential expression analysis

Transcript abundances were quantified by pseudo-aligning RNA-seq reads to either a reference genome (Harrison *et al.* 2018) or to a *de novo* assembly (He 2018) using Salmon v0.9.1 (Patro *et al.* 2017). We used tximport v1.12.3 (Soneson *et al.* 2016) in conjunction with DESeq2 v1.24.0 (Love *et al.* 2014) to model gene-level estimated counts while correcting for changes in transcript usage across samples. Differential expression was considered to be significant when fold changes were greater than 2 for pairwise Wald contrasts of treatments, with a FDR-corrected *p* value of less than 0.05. The mean of the normalized counts for each gene was used as the informative covariate for independent hypothesis weighting (Ignatiadis *et al.* 2016) in order to optimize the power of multiple testing. Immune genes were identified using an in-house prepared gene list based on an HMMER-based approach and Uniprot Blastp (He 2018; UniProt Consortium 2018).

Survival following lethal infection

In total, 175 conventional and 169 germ-free *B. germanica* males from 3 different cohorts were used. The treatments were 1) infection with a lethal dose of *P. entomophila* (2.0×10^5 CFUs per individual); 2) wounding control (injection with Ringer's solution); and 3) unmanipulated control. For information on the *P. entomophila* strain and its cultivation and preparation see '**Chapter I – Material and methods: Insects and bacteria**' and '**Chapter I – Material and methods: Bacterial inoculation**'. The exact assignment to the treatments by cohort is shown in Tab. 4.1. After the treatment the cockroaches were individually reared, supplied with water

ad libitum and the survival was recorded until no more deaths occurred, which was after 62.5 h. Bacterial dose was determined in pre-experiment injection assays.

Tab. 4.1: Assignment of conventional and germ-free cockroaches by cohort to the different treatments (injection with 1.5 x 10^5 *P. entomophila* CFUs, wounding control (injection with Ringer's solution) and unmanipulated control). The dose was determined in pre-experiment injection assays.

Cohort	Treatment	Number of males
A	Conventional infected	45
	Germ-free infected	41
	Conventional wounded	10
	Germ-free wounded	10
	Conventional unmanipulated	5
	Germ-free unmanipulated	5
B	Conventional infected	36
	Germ-free infected	34
	Conventional wounded	10
	Germ-free wounded	10
	Conventional unmanipulated	5
	Germ-free unmanipulated	5
C	Conventional infected	34
	Germ-free infected	34
	Conventional wounded	15
	Germ-free wounded	15
	Conventional unmanipulated	15
	Germ-free unmanipulated	15

The survival of conventional and germ-free cockroaches following lethal infection was analysed using Cox proportional hazard models with cohort as a random factor in R 3.6.1 (R Core Team 2019) with the package coxme v2.2-14 (Therneau 2019). Comparisons among treatment levels were carried out with post-hoc Tukey tests using a Bonferroni correction, using the package multcomp v1.4-10 (Hothorn *et al.* 2008). Median survival time for each treatment was calculated using the survival package v2.44-1.1 (Therneau 2015) and the survminer package v0.4.6 (Kassambara *et al.* 2019).

Results

Sterility testing by plating and 16S sequencing

Results are reported in **Chapter II**.

Comparison of immune gene regulation in conventional and germ-free cockroaches

Using the reference genome (Harrison *et al.* 2018) 25451 putative genes were identified and 184 of those were significantly different expressed between conventional and germ-free cockroaches (Fig. 4.1A). In comparison, when the *de novo* assembly (He 2018) was used 111778 putative genes were identified and 1082 of those were significantly different expressed between conventional and germ-free cockroaches (Fig. 4.1B). Additionally, independent of the alignment method used, the expression profile of all genes and immune-related genes is clearly categorized by the presence of the microbiota according to principle component analyses (Fig. 4.2).

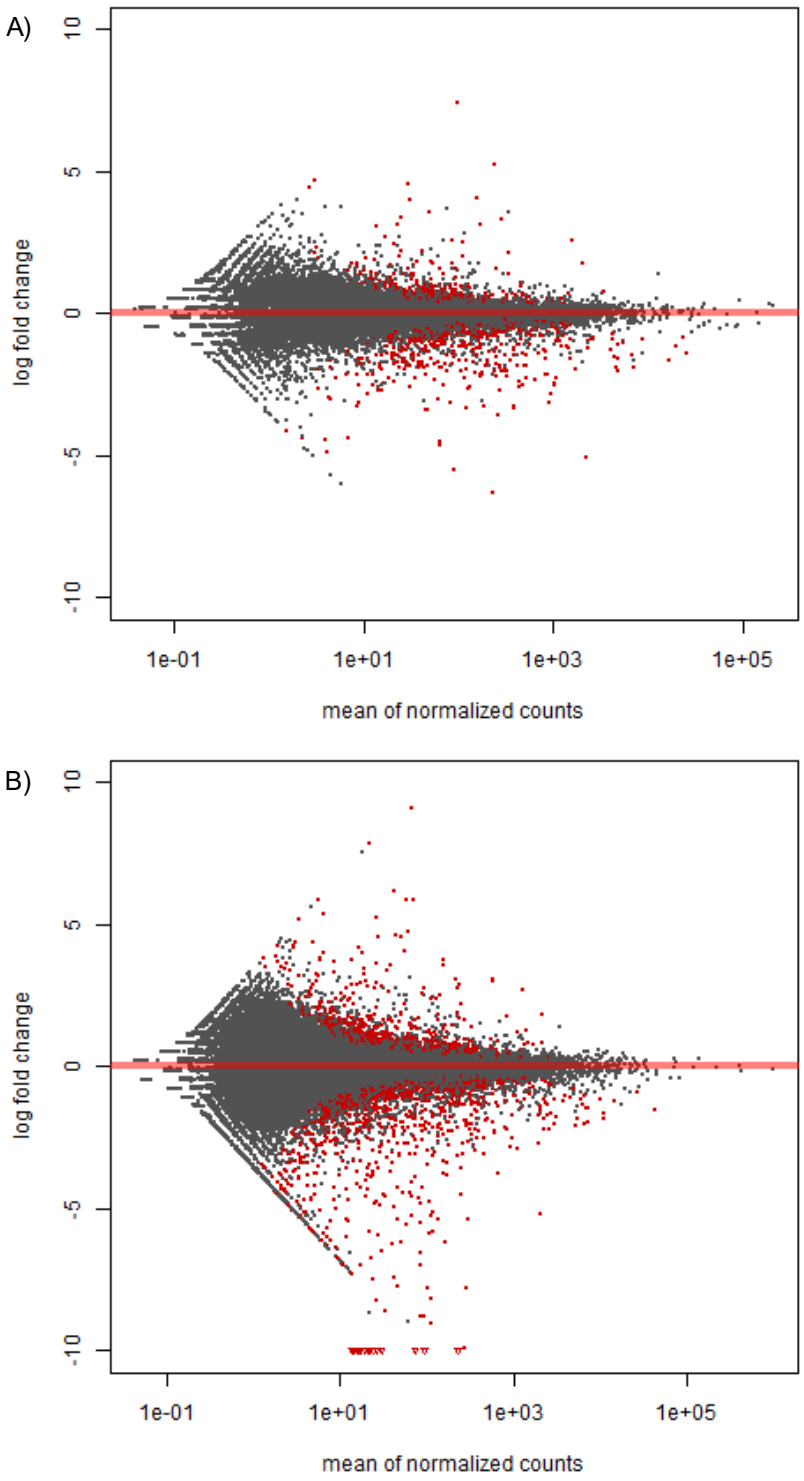

Fig. 4.1: MA plots of expressed genes in conventional and germ-free cockroaches: **A)** when the published genome (Harrison *et al.* 2018) was used as a reference for the alignment; **B)** when the *de novo* assembly (He 2018) was used as a reference for the alignment. Genes which are differentially expressed between the two groups are marked in red. Genes which are not significantly different expressed are marked in grey and the mean of normalized counts is represented by the red line. Differential expression analysis was performed by DEseq2 v1.24.0.

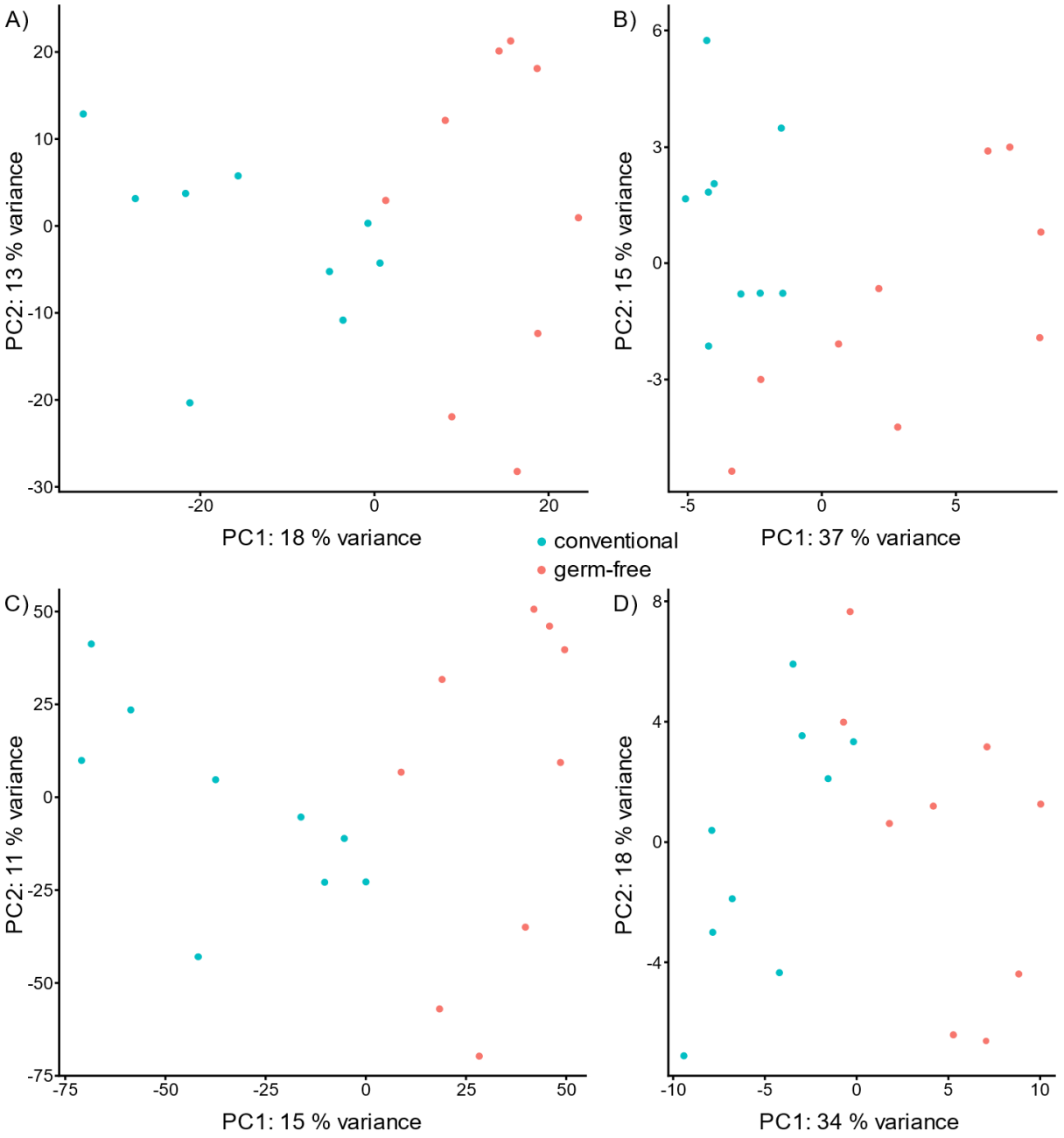

Fig. 4.2: Principal component analyses of **A)** all genes when the published genome (Harrison *et al.* 2018) was used as a reference for the alignment; **B)** immune-related genes when the published genome was used as a reference for the alignment; **C)** all genes when the *de novo* assembly (He 2018) was used as a reference for the alignment; **D)** immune-related genes when the *de novo* assembly was used as a reference for the alignment. Conventional *B. germanica* males are represented in light blue and germ-free ones in light red.

Involvement in immune-related task was attributed to 294 genes when the reference genome was used for the alignment and 19 of them were differentially expressed in conventional and

germ-free cockroaches (Fig. 4.3). Twelve hemolymph lps-binding protein (lectins) related genes were upregulated in germ-free cockroaches, while 2 were upregulated in conventional cockroaches. In conventional cockroaches, 2 tenecin-1 genes, a peroxiredoxin-6, a Niemann-Pick disease type C intracellular cholesterol transporter 2 (NPC2) and a beta-1,3-glucan-binding protein gene were upregulated compared to germ-free ones as well.

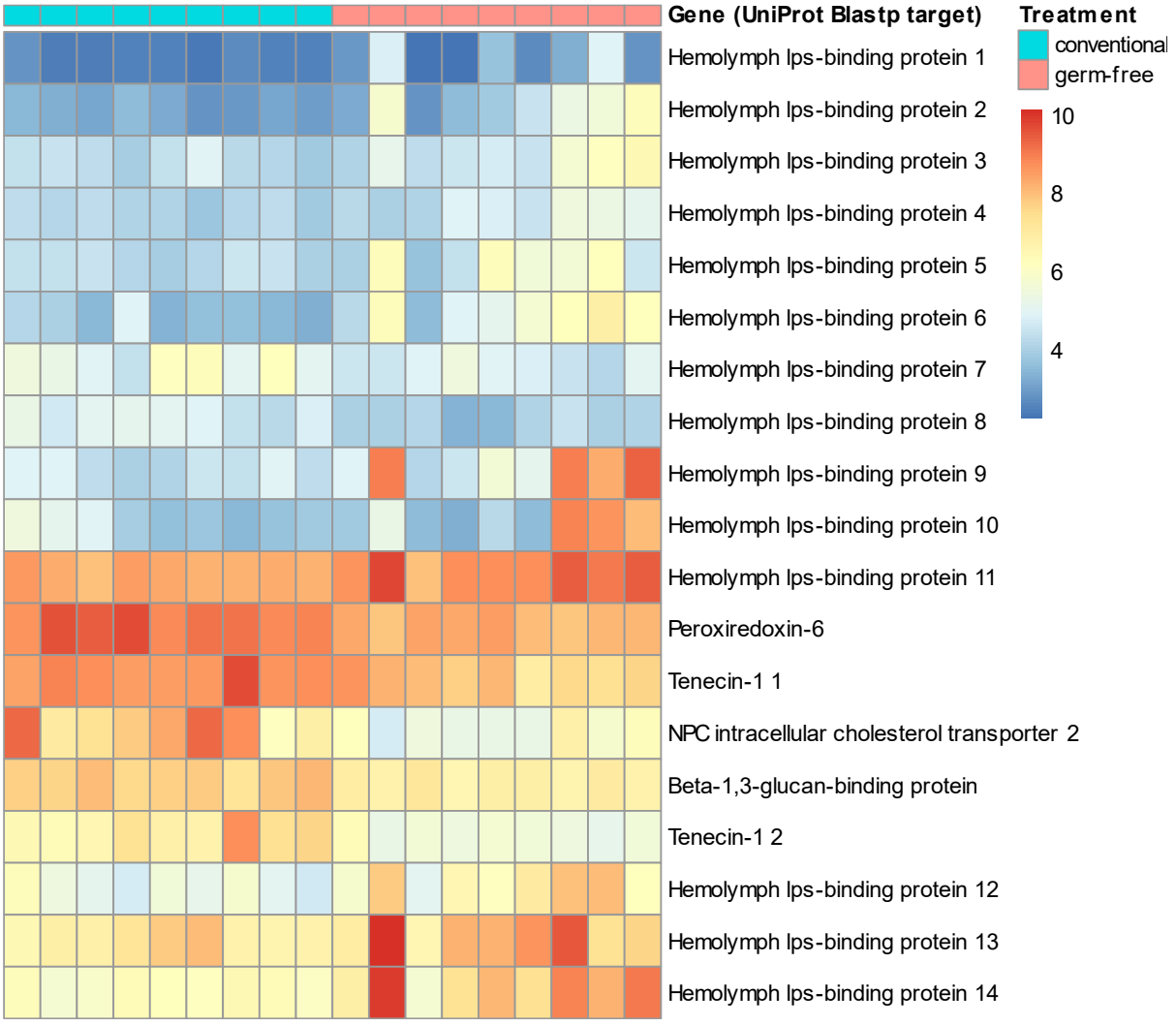

Fig. 4.3: Heatmap of differentially expressed immune-related genes (UniProt Blastp target; UniProt Consortium 2018) in conventional and germ-free cockroaches according to a DESeq2 v1.24.0 analysis when the published genome (Harrison *et al.* 2018) was used as a reference for the alignment.

In comparison when the *de novo* annotation was used for the alignment 309 genes were identified as immune-related genes and 30 of them were differentially expressed in

conventional and germ-free cockroaches (Fig. 4.4). Eleven hemolymph lps-binding protein related genes were upregulated in germ-free cockroaches, while 5 were upregulated in conventional cockroaches. In conventional cockroaches, 3 tenecin-1 genes, a peroxiredoxin-6, a NPC2 and a beta-1,3-glucan-binding protein gene, were upregulated compared to germ-free ones. Genes which are exclusively found and upregulated in conventional cockroaches when the *de novo* annotation was used for the alignment were a transferrin, a caspase-1, a macrophage mannose receptor (MMR) 1, an alpha-1-macroglobulin, a PGRP-SD, a lysozyme c-1 and a catalase.

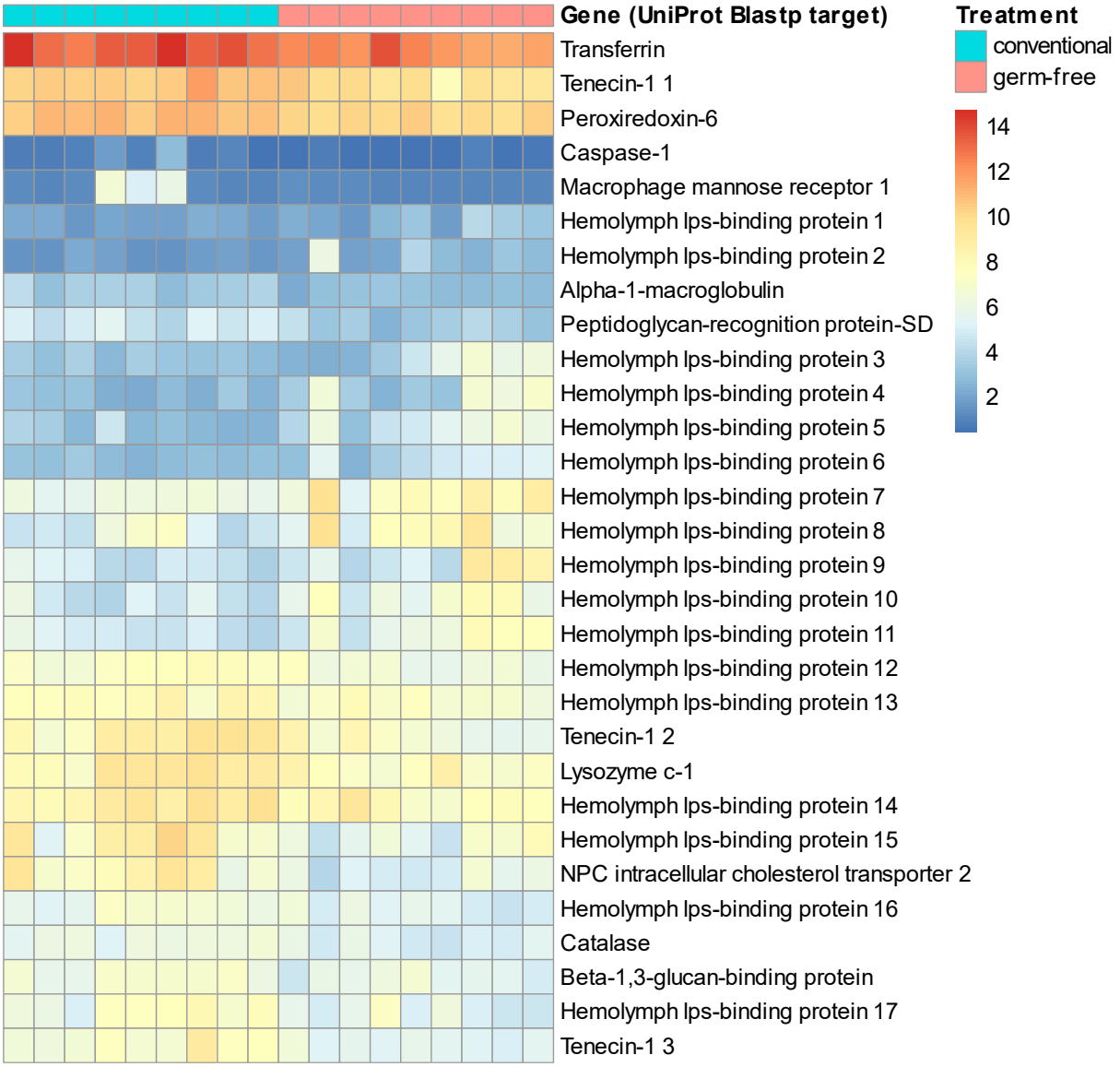

Fig. 4.4: Heatmap of differentially expressed immune-related genes (UniProt Blastp target; UniProt Consortium 2018) in conventional and germ-free cockroaches according to a DESeq2 v1.24.0 analysis when the *de novo* assembly (He 2018) was used as a reference for the alignment.

Survival following lethal infection

Unmanipulated and wounded (injected with Ringer's solution) conventional *B. germanica* males showed no or neglectable mortality (conventional wounded: 2.9 %) over the course of the experiment, whereas their germ-free counterparts showed low, but not significantly different, mortality (germ-free unmanipulated: 8.0 %; Cox proportional hazard regression: conventional unmanipulated vs. germ-free unmanipulated: $z = 0.849$, $p > 0.1$; germ-free

wounded: 17.1 %; Cox proportional hazard regression: conventional wounded vs. germ-free wounded: z = 1.557, $p > 0.1$; Fig. 4.5). When injected with a lethal dose of *P. entomophila*, germ-free males died faster than their conventional counterparts. Their median survival time was 18.5 h, whereas the median survival time for conventional males was 36.5 h. Furthermore, by the end of the experiment germ-free males showed a significantly higher mortality (88.1 %) compared to the conventional ones (66.1 %; Cox proportional hazard regression: z = 6.040, $p < 0.001$).

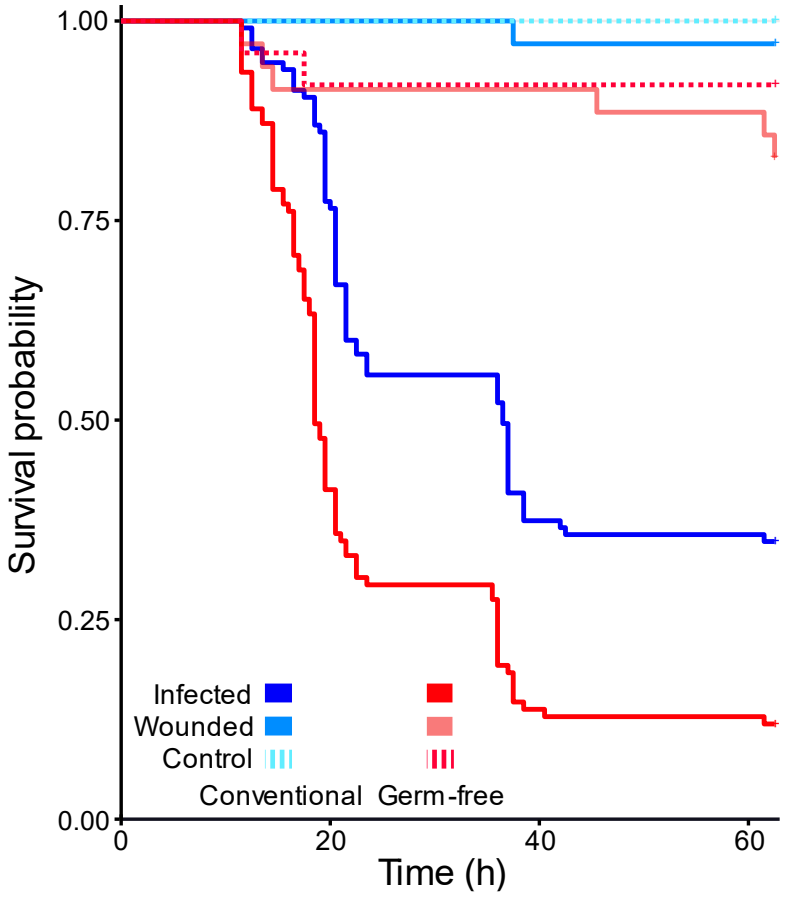

Fig. 4.5: Kaplan-Meier survival curves for conventional and germ-free *B. germanica* males being unmanipulated (control), wounded (injected with Ringer's solution) or infected with a lethal dose of *P. entomophila*.

Discussion

The methods used to quantify transcript abundances at least slightly influenced the outcome of the analyses. When the *de novo* assembly (He 2018) was used for the alignment 86327 more genes were identified compared to the method using the reference genome (Harrison *et al.* 2018) for the alignment (111778 vs. 25451 genes, respectively). The number of genes which were identified as being differentially expressed between conventional and germ-free *B. germanica* males was influenced by the alignment approach as well. In total 184 genes were identified as being significantly different expressed when the genome was concerned as a reference (Fig. 4.1A) while 1082 were identified when the *de novo* assembly was concerned (Fig. 4.1B). These findings were emphasized by microbiota-dependent gene expression patterns for every gene analysed (Fig. 4.2A and 4.2C), including immunity-related genes (Fig. 4.2B and 4.2D). Additionally, it was found that a transferrin, a caspase-1, a MMR 1, an alpha-1-macroglobulin, a PGRP-SD, a lysozyme c-1 and a catalase are only considered to be differentially expressed in conventional and germ-free cockroaches when the *de novo* assembly is used in for the alignment (Fig. 4.3; Fig. 4.4). An explanation for the general higher gene numbers when the *de novo* assembly is used could be redundancy in the *de novo* assembly caused by alleles, paralogs or fragmentation. Reasons further explaining these discrepancies and the differentially expressed genes being additionally found in the analysis based on the *de novo* assembly might be that either genes are missing in the reference genome due to the gene prediction method used for its annotation or that some transcripts in *de novo* assembly are likely not of host origin but microbial contaminants. Nevertheless, beside these uncertainties it is arguably the best approach to combine the findings of both analyses especially because they also feature a great overlap. This also highlights the need for researchers to use multiple assemblies for their analyses whenever they are available.

Combining both analyses, germ-free cockroaches showed upregulation for 11 (*de novo* assembly) and 12 (reference genome) hemolymph lps-binding protein related genes while conventional ones showed upregulation for only 2 (reference genome; Fig. 4.3;) and 5 (*de novo* assembly; Fig. 4.4). It has been shown that the hemolymph of *P. americana* cockroaches contains various proteins encoded by those genes which might induce the intracellular transport of molecules featuring sugar moieties (Kawasaki *et al.* 1996). Another possible function is that they are important for trapping *Blattabacterium sp.* endosymbionts that have leaked from the fat body into the hemolymph while only a small number of them might also stimulate the defence mechanism against foreign microbes (Jomori *et al.* 1990; Kawasaki *et al.* 1996). The latter is further proven by the lack of upregulation of antimicrobial effector genes in germ-free cockroaches in both analyses.

Genes involved in the control of microbes beside the *Blattabacterium sp.* endosymbionts are only upregulated in conventional cockroaches. These are 2 (reference genome) and 3 (*de novo* assembly) tenecin-1 genes (Fig. 4.3; Fig. 4.4) belonging to the insect defensin family. They encode for proteins which's C-terminal β-helical sheets display potent antimicrobial activity especially against Gram-positive bacteria and less against fungi and Gram-negative bacteria and they potentially act on the pathogen membrane (Lee *et al.* 1998). Further, there are additional genes which directly combat microbes upregulated in conventional *B. germanica* males when the analysis using the *de novo* assembly is concerned, namely a transferrin, a caspase-1, a alpha-1-macroglobulin, a lysozyme c-1 and a catalase (Fig. 4.4). Transferrins are broadly considered to be involved in iron storage and transport in insects (Lowenberger 2001). It could be shown that transferrins are upregulated upon infection in various insects including *D. melanogaster*, *A. aegypti* and *Mastotermes darwiniensis* (Thompson *et al.* 2003). It has been assumed that they take free iron away from pathogens which require it for growth and development and thereby reducing their infectious potential or being involved in the downstream production of AMPs (Lowenberger 2001; Thompson *et al.* 2003; Harizanova *et al.* 2005). Caspases are a family of cysteine proteases which are involved in apoptosis but there is also growing evidence that they are involved in immunity as well (Jearaphunt *et al.* 2014). In crayfish caspase-1 regulates phenoloxidase activity in response to bacterial infections by cleavage of prophenoloxidase (proPO; Jearaphunt *et al.* 2014). Additionally, in insects caspases are involved in the resistance against nuclear polyhedrosis virus infections in the case of caspase-1 in *B. mori* (Qin *et al.* 2012) or like Dredd in *Drosophila* regulating the expression of AMPs upon bacterial infection (Leulier *et al.* 2000). Protease inhibitors like the alpha-1-macroglobulin are present in all eukaryotes and they limit the activity proteases including digestive enzymes, the components of signalling cascades and pathogen-encoded virulence factors (Gubb *et al.* 2010). The inhibitory action is due to forming macromolecular cages around the proteases in which they are crosslinked and trapped (reviewed in Zhao *et al.* 2012). In *Drosophila* fruit flies macroglobulins are upregulated following infection and they bind specifically to fungi and bacteria to stimulate phagocytosis (reviewed in Gubb *et al.* 2010). Lysozyme c-1 is a chicken or conventional type (C-type) lysozyme (reviewed in Kajla *et al.* 2011). In the animal kingdom three major different types differing in their amino acid sequences, in their biochemical as well as in their enzymatic properties have been described namely the C-type, the goose-type and the invertebrate type (reviewed in Prato 2014). Lysozymes were the first antimicrobial factors which were isolated from insect hemolymph (reviewed in Boman and Hultmark 1987) and they have been identified in several insect species resembling the C-type (reviewed in Wilson and Ratcliffe 2000). They cleave β(1-4) bonds between N-acetylmuraminic acids and N-acetylglucosamine in the cell walls of bacteria (Urich 1994). In the cockroach *Blaberus discoidalis*, for example, the injection of bacteria and

yeast leads to an increase of bacteriolytic lysozyme in the hemolymph which also favour phagocytic activity (Wilson and Ratcliffe 2000). In addition, immune receptor genes are differentially expressed in conventional and germ-free cockroaches as well. A gene which encodes for a beta-1,3-glucan binding protein is found in both analyses (Fig. 4.3; Fig. 4.4). Beta-1,3-glucan binding proteins are pattern-recognition proteins that bind to microbial beta-1,3-glucans which are found inside the cell wall especially of fungi they trigger proPO activation and AMP synthesis (Rolff and Reynolds 2009). For example, in the cockroach *B. discoidalis* a beta-1,3-glucan specific lectin activates the proPO cascade. In *T. molitor* beetles a beta-1,3-glucan binding protein, namely GNBP3, can activate the proPO cascade as well but it can also induce the Toll signalling pathway (Yang *et al.* 2018). This might connect to the mentioned tenecin-1 overexpression since GNBP3 induces downstream tencin-1 gene expression in fungi infected *T. molitor* (Yang *et al.* 2018). The in both analyses upregulated NPC2 gene (Fig. 4.3; Fig. 4.4) in conventional cockroaches might be involved in microbial recognition, too. In vertebrates, proteins encoded by members of this gene group participate in lipid metabolism and innate immune signalling (Inohara and Nuñez 2002; Bryant *et al.* 2010). In addition, Shi *et al.* (2012) could show that NPC2 variants bind to the bacterial cell wall components lps, lipid A, peptidoglycan and lipoteichoic acid. This binding leads to an overexpression of the NPC2 genes which further results in the production of the AMP diptericin (Shi *et al.* 2012). Again, in the same manner as for the genes directly fighting microbes there are immune receptors exclusively found the analysis based on the *de novo* assembly being upregulated in conventional cockroaches, namely MMR 1 and PGRP-SD (Fig. 4.4). MMRs like MMR 1 play an important role in pattern recognition of microbes by recognising carbohydrates on the surface of a wide range of yeasts, parasites, Gram-negative and Gram- positive bacteria and thus mediating endocytosis and phagocytosis of microbes (Stahl and Ezekowitz 1998). PGRP-SD belongs to the class of PRRs which bind and recognise bacterial peptidoglycan (reviewed in Feldhaar and Gross 2008). In *D. melanogaster* PGRP-SD participates in the recognition of Gram-positive bacteria by forming a complex with PGRP-SA and GNBP1 (Bischoff *et al.* 2004; Wang *et al.* 2008).

Together with that, a homolog of the mammalian peroxiredoxin-6 gene was overexpressed in conventional *B. germanica* males in both analyses (Fig. 4.3; Fig. 4.4). It belongs to a family of antioxidants which protect cells from metabolically produced reactive oxygen species (ROS; Robinson *et al.* 2010). On the one hand, it has been shown that ROS can efficiently kill invading bacteria in insects and that *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus bombyseptieus* infections increase ROS levels in *B. mori* (Hu *et al.* 2013; Zhang and Lu 2015; Zhang *et al.* 2015; Chen and Lu 2018). In addition, the gene catalase is also upregulated in conventional *B. germanica* males when only the *de novo* assembly is

concerned. Catalase occurs universally in insects and it protects cells from ROS, too. It degrades the ROS hydrogen peroxide into oxygen and water (reviewed in Felton and Summers 1995).

All these exclusive upregulations of immune-related genes in conventional *B. germanica* males together with the already mentioned studies further showcase, that the host's immune system drastically maintains the coexistence of insects and their microbiota. Here, these genes are very likely upregulated to keep the cockroach's microbiome in abundance, composition and location under control (Douglas 2014). For example, in *Drosophila* only the Imd pathway is expressed in the midgut of the adult fly presumably to suppress, but not eliminate, the populations of symbiotic bacteria via AMP production (Buchon *et al.* 2009; Douglas 2014). Additionally, as also indicated in our findings, ROS species, which are produced in the midgut of *Drosophila* and *A. aegypti* mosquitoes via the membrane-associated dual oxidase (DUOX), may play a central role in the control of the gut microbiota (Douglas 2014). In *A. aegypti* this is indicated by an overgrowth of the bacterial populations when DUOX is silenced by RNAi or after the insect takes a blood meal which reduces the DUOX activity as well (Oliveira *et al.* 2011). Contrary, in Drosophila DUOX is only activated when yeasts or pathogenic bacteria are ingested, but not by the symbiotic gut bacteria (Ha *et al.* 2009a; Lee *et al.* 2013). However, basal ROS production still occurs, and it is critical, because otherwise the gut microbiota will overgrow in an uncontrolled manner (Ha *et al.* 2009b).

These fine-tuned immune responses may also attribute to the observation, that conventional male *B. germanica* cockroaches survive an infection with the Gram-negative soil bacterium *P. entomophila* much better than their germ-free counterparts (Fig. 4.5). Compared to germ-free cockroaches conventional ones showed a 22 % higher survival after infection. The results from another study where *B. germanica* cockroaches were also more susceptible to an infection by the fungus *Metarhizium anisopliae* after depletion of their gut microbiota through the feeding of antibiotics (Zhang *et al.* 2018) corroborate our work. Taken together it is indisputable that the host associated microbiota plays a crucial role in the pathogen defence of *B. germanica* males, but the reasons for that are less straightforward.

First, it potentially takes less effort to elicit a potent immune reaction to combat an invading pathogen when the host immune system is constantly basally stimulated by the inherent microbiota, as already shown by the upregulation especially of immune effectors in conventional cockroaches. It is further supported by several studies. For example in the mosquitos *A. aegypti* (Xi *et al.* 2008) and *A. gambiae* (Dong *et al.* 2009) as well as in tsetse flies (Wang *et al.* 2009) it is showcased that antibacterial responses against midgut microbiota in the mosquito and symbiotic bacteria in the tsetse fly also protect the host against infection

by viruses and parasites, thereby interrupting the transmission cycle of these vector borne pathogens. Findings which are further supported by a study on *D. melanogaster* (Sansone *et al.* 2015) showing that antiviral immunity is enhanced through the gut microbiota signalling the NF-kB pathway and by a study on honeybees (Kwong *et al.* 2017) which could show that the gene expression of the AMPs apidaecin and hymenoptaecin in gut tissue and in the hemolymph is stimulated when the microbiota is present. This resulted in an improved survivorship following *E. coli* injection.

Second, the microbiota might directly combat the *P. entomophila* infection by producing antimicrobial compounds like AMPs and ROS or by colonization resistance. For instance, microbiota in the crop of honeybees especially the bacterium *Lactobacillus kunkeei* show potent antimicrobial properties against a variety of microorganisms (Vásquez *et al.* 2012). Additionally, the gut bacterium *Enterococcus mundtii* of the lepidoptera *S. littoralis* produces the AMP mundticin KS which can impair pathogen colonization of the host (Shao *et al.* 2017). Further, ROS produced by the gut bacterium *Enterobacter sp.* in the mosquito *A. gambiae* depresses *Plasmodium* infection (Cirimotich *et al.* 2011). Likewise, an example for colonization resistance is given by a study on the desert locust *S. gregaria* where individuals which harboured a more complex gut microbiota were less susceptible to an infection with the bacterium *S. marcescens* showing, that species-rich communities are more resistant to invasion (Dillon *et al.* 2005). There is also further evidence that the immunocompetence of insects against parasitoids and entomopathogenic fungi can be enhanced by certain hemolymph microorganisms (reviewed in Blow and Douglas 2019). Nevertheless, to prove if the microbiota of *B. germanica males* is involved in the direct combat of pathogens being introduced into the hemolymph as well the transcriptome of infected conventional and germ-free cockroaches needs to be compared and refaunation experiments need to be conducted.

Third, it might be possible as well that the removal of the microbiota is very detrimental for the host in general, especially if symbionts are involved in certain metabolic functions like provision of nutrients (Futo *et al.* 2016; reviewed in the general introduction). This might lead to a generally poorer body condition which may result in a lack of immunocompetence necessary for building up a sufficient immune response (Futo *et al.* 2016).

In conclusion, we could show that several immune-related genes are differentially expressed between conventional and germ-free uninfected *B. germanica* males. This stimulation of the immune system by the indigenous microbiota might help fighting pathogen infections as well, as shown by a reduced survival upon *P. entomophila* infection of germ-free cockroaches. Whether the microbiota directly combats invading microbes including pathogens remains to be tested via further transcriptome analyses. Furthermore, refaunation experiments with certain

microbial taxa would be very useful to attribute functions on immunity to single candidate taxa. Overall, our findings support the concept of microbes being ubiquitous in insects and having pervasive impacts on multiple aspects of insect's biology, especially on nutrition and immunity (Douglas 2015). Most importantly, our study broadens the taxonomic spectrum and emphasises thereby the general importance of microbes on insect life history, since studies to date focused exclusively on a number of prominent model insects. The importance of examining non-model insect species as well becomes immediately apparent when re-examining one of our findings. As already mentioned, we could show that in our cockroach system the Toll pathway seems to be involved in controlling the indigenous microbiota whereas in *Drosophila* it is the Imd pathway instead. In general, this finding indicates that the raw concepts for maintaining the coexistence of insects and their microbiota are conserved between taxa, but the mode of action is taxa or at least order specific.

Summary

Traditionally, philosophy uses the discrimination of self and non-self to define individuality with the immune system performing this discrimination. In the evolutionary field of biology this distinction is not that simple. Nowadays it is becoming more and more apparent that individuals can no longer be considered as 'lone isolated islands' in the 'environmental sea'. All kinds of eukaryotic taxa harbour their own microbiota consisting of bacteria, archaea, fungi, protozoa and viruses and they are tolerated by the host's immune system because of their manifold beneficial functions on, for example, host nutrition, detoxification, development, fecundity or pathogen protection. However, not only the beneficial microbiome, but also the host's nutrition can strongly affect its physiology and its ability to combat pathogen infections. Microbiome and host form a unit – the holobiome. Notably, even though we gained insights on either the function of the microbiome or of the nutrition on host immune defence in diverse separate studies we still poorly understand how they act together in particular organisms. An insect model system to study these interactions are cockroaches. This is because, 1) they are omnivorous generalists, which makes them easily accessible for nutritional studies; 2) they harbour a diverse microbiota which can be manipulated through sterilization methods; and 3) they feature effective strategies to combat pathogens since they are frequently exposed to a rich antigenic environment due to their lifestyle.

First, in **Chapter I** I investigated the nutritional dependencies of immunity in the cockroach system by performing food choice experiments using the cockroach species *B. orientalis* upon exposure to the opportunistic Gram-negative bacterial insect pathogen *P. entomophila*. I could show that depending on the strength of infection *B. orientalis* males reduce their overall nutrient intake and increase the P:C ratio being consumed. Interestingly, these behavioural shifts do not boost the insect's immunity as indicated by the examination of the hemolymph's antimicrobial activity, the abundance of immune proteins in the hemolypmph or the general host survival. This lack of benefits for the host highlights a possible decoupling of dietary macronutrient regulation from immunity in these invasive animals with the possibility that anorexia, in general, might be a more powerful tool if diet quality is highly unpredictable for generalist species.

In **Chapter II** I evaluated two different approaches for the development of a germ-free *B. germanica* cockroach breeding system which forms the basis of any study dealing with the function of the cockroach microbiome. While one of these methods uses peracetic acid, the other one uses a combination of peracetic acid and sodium hypochlorite to surface-sterilize cockroach oothecas to deprive the hatchlings from their natural microbiota. These treatments

should leave them only with their obligate symbiont *Blattabacterium sp.*, which supplies essential vitamins and is required for the development into fecundant adults. I tested the success of those techniques by plating adult individuals on LB-agar and by using state of art 16S metabarcoding. It turned out that both methods performed quite poorly leading to individuals which can be considered as germ-free in 40 % of all cases. I therefore developed our own method by combining sequential ootheca surface sterilizations with peracetic acid and sodium hypochlorite followed by a treatment of freshly hatched nymphs with the antibiotics rifampicin and gentamicin which significantly improved its effectiveness resulting in germ-free adult cockroaches to 99 % of all cases. In addition, I used our germ-free cockroach system for an early study on the impact of the absence of an intact microbiome on developmental time. I could show that *B. germanica* cockroaches deprived of their natural microbiota needed approximately 35 days longer from the day of hatching to the day molting into adults than their conventional counterparts, which already grants a small glimpse on the strong impacts of the microbiome on the host physiology and its overall performance.

In **Chapter III** I analysed the transcriptome of germ-free and conventional *B. germanica* males and followed their survival upon *P. entomophila* systemic infection to gain further insights on the influence of the cockroach microbiome on host traits. The basis of our gene identification were two published genomes either the one by Harrison *et al.* (2018) or the one by He (2018). Depending on the reference genome used for the analyses small differences existed. When the Harrison *et al.* genome was used 25451 putative genes were identified and 184 of those including 19 immune-related genes were significantly different expressed between conventional and germ-free cockroaches. When the He genome was used 111778 putative genes were identified and 1082 of those including 30 immune-related genes were significantly different expressed between conventional and germ-free cockroaches. Immune-related genes which were significantly expressed between germ-free and conventional cockroaches identified with both reference genomes included hemolymph lps-binding protein related genes which were mostly upregulated in germ-free individuals because of their role in trapping the *Blattabacterium sp.* endosymbiont and tenecin-1 genes, a transferrin, a caspase-1, a alpha-1-macroglobulin, a lysozym c-1 as well as a catalase found to be upregulated in conventional individuals. All the latter ones contribute to the recognition and the suppression of microbial life to maintain a stable host microbiota. This regulation of gene expression by the microbiome might also assist the host in combating infections as indicated by the significantly higher survival of conventional cockroaches infected with *P. entomophila*.

In conclusion I was able to show, that host biology is heavily shaped by microbial life. Those microbes can be either invading pathogens or commensal or beneficial microbiota. In all cases they radically alter host behaviour, development and/or physiology. While pathogens only harm

their hosts, the microbiome promotes host phenotypes like development or immune competence. Therefore, pathogens do not only interact with their hosts but also with its microbiota. Since this fact became only apparent within the last few years more research is needed to reveal all its aspects. A stable foundation for such future work is paved by my recently established germ-free *B. germanica* breeding system. In this framework it will be particularly important and likewise exciting to perform refaunation experiments with single microbial taxa followed by infections with different pathogens and further transcriptomic analyses to uncover their special tasks in this broad network of interactions.

Zusammenfassung

In der Philosophie wird traditionell zwischen selbst und nicht-selbst (fremd) unterschieden. Dabei sind Abläufe im Immunsystem von Bedeutung, um Individualität zu definieren. Evolutionsbiologisch ist eine solche Unterscheidung jedoch weitaus schwieriger. So zeigt aktuelle Forschung, dass Individuen nicht als isoliert in ihrer umgebenden Umwelt gesehen werden dürfen. Eukaryotisches Leben wird von einer Vielzahl mikrobiellen Lebens, dem Mikrobiom, welches Bakterien, Archaeen, Pilze, Protozoen und Viren miteinschließt, bewohnt. Die Mikroorganismen haben meist nützliche Funktionen für den Wirt, sie sind beispielsweise wichtig bei der Ernährung, der Entgiftung, der Individualentwicklung, der Fortpflanzung oder der Immunabwehr. Mikrobiom und Wirt bilden eine Einheit, dass Holobiom.

Jedoch nicht nur das Mikrobiom beeinflusst Physiologie und Immunabwehr des Wirts, auch die Ernährung des Wirts kann sich positiv auswirken. Beide Aspekte (Mikrobiom und Ernährung) wurden bisher ausgiebig unabhängig voneinander untersucht, nicht jedoch, wie sie zusammenwirken. Ein geeignetes Untersuchungsobjekt, um diese Zusammenhänge besser zu verstehen, sind Schaben, da sie 1) omnivore Generalisten sind, wodurch sie sich besonders für Ernährungsstudien eignen; 2) eine diverse mikrobielle Flora beherbergen, die sich durch Sterilisationsmethoden manipulieren lässt; und 3) weil sie effektive Strategien zur Bekämpfung von Krankheitserregern besitzen, da sich Schaben häufig in antigenreicher Umgebung aufhalten.

In **Kapitel I** habe ich anhand von Futterauswahlversuchen mit der Schabenart *B. orientalis* den Einfluss der Ernährung auf die Immunabwehr während einer Infektion mit dem opportunistischen Gram-negativen Bakterium *P. entomophila* untersucht. Dabei konnte ich zeigen, dass abhängig vom Ausmaß der Infektion *B. orientalis* Männchen ihre Nahrungsaufnahme vermindern dabei sich jedoch das Verhältnis von Protein zu Kohlenhydrat in der aufgenommenen Nahrung zu Gunsten des Proteinanteils erhöht. Interessanterweise beeinflussen diese Verhaltensänderungen weder die die Konzentration von antimikrobiellen Peptiden in der Hemolymphe noch ihre antimikrobielle Aktivität. Auch ein Einfluss auf die krankheitsbedingte Mortalität der Schaben ist nicht erkennbar. Deshalb deutet alles auf die Entkopplung von ernährungsbedingter Makronährstoffregulierung und Immunabwehr in dieser Insektenart hin. Folglich vermag Anorexie ein machtvolleres Instrument im Kampf gegen Krankheitserreger sein, wenn die Qualität der zur Verfügung stehenden Nahrung, wie es bei Generalisten und als solchen auch Schaben der Fall ist, unvorhersehbar ist.

In **Kapitel II** evaluierte ich zwei verschiedene Ansätze zur Etablierung einer mikrobenfreien *B. germanica* Zuchtlinie, welche den Grundstein für Studien zur Funktion des Mikrobioms bei

Schaben liefert. Einer der Ansätze beruhte dabei auf den Einsatz von Peressigsäure und der andere auf einer Kombination von Peressigsäure und Natriumhypochlorit zur Oberflächensterilisation von Ootheken. Diese Behandlungen sollten alle Mikroorganismen, die die Schaben besiedeln bis auf den obligaten Symbionten *Blattabacterium sp*., welcher essentielle Vitamine bereitstellt und für die vollständige Individualentwicklung zum geschlechtsreifen Tier von Nöten ist, abtöten. Ich testete den Erflog beider Behandlungen durch Ausplattieren von erwachsenen Tieren auf LB-Agar und mit Hilfe neuster 16S Metabarcoding Sequenziermethoden. Es zeigte sich, dass beide Methoden keine zufriedenstellenden Ergebnisse lieferten, da sie nur in 40 % der Fälle mikrobenfreie Tiere lieferten. Folglich entwickelte ich meine eigene Methode zur Etablierung einer mikrobenfreien *B. germanica* Zuchtlinie. Ich kombinierte dabei den Sterilisationsprozess durch Peressigsäure und Natriumhypochlorit mit einer Antibiotikabehandlung mit Rifampicin und Gentamicin der frisch geschlüpften Schaben. Diese neue Behandlungsmethode erhöhte die Erfolgsquote auf 99 %. Zusätzlich nutzte ich die mikrobenfreien Schaben für eine Studie, den Einfluss des Mikrobioms auf die Individualentwicklung zu untersuchen. Dabei konnte ich zeigen, dass mikrobenfreie Schaben der Art *B. germanica* ca. 35 Tage länger für die Entwicklung hinzu erwachsenen Tieren benötigen. Der große Stellenwert des Mikrobiom in der Biologie dieser Tiere wurde dadurch deutlich.

Um weitere Einblicke für die Funktion des Mikrobioms zu erlangen, analysierte ich in **Kapitel III** das Transkriptom von mikrobenfreien und konventionellen *B. germanica* Männchen. Ich verglich ebenfalls den Sterbeverlauf beider Gruppen nach einer systemischen Infektion mit *P. entomophila*. Die Grundlagen meiner Genidentifikation lagen dabei auf veröffentlichtem Schabengenom von Harrison *et al.* (2018) und von He (2018). Es ergaben sich aber abhängig vom verwendeten Referenzgenom kleine Unterschiede in den Analysen. Wenn das Genom von Harrison *et al.* als Referenz herangezogen wurde, konnten ich 25451 putative Gene identifizieren, von denen 184, inklusive 19 Immungenen, zwischen mikrobenfreien und konventionellen Schaben unterschiedlich exprimiert wurden. Wurde im Vergleich das Genom von He zugrunde gelegt, konnte ich 111778 putative Gene identifizieren, von denen 1082, inklusive 30 Immungenen, zwischen mikrobenfreien und konventionellen Schaben unterschiedlich exprimiert wurden. Unterschiedlich exprimierte Immungene, die dabei mit beiden Ansätzen identifiziert wurden, waren: Hemolymph-lps-binding-Protein-Gene, welche besonders in den mikrobenfreien Schaben überexprimiert waren um den Symbionten *Blattabacterium sp.* zu regulieren; bzw. Tenecin-1 Gene, ein Transferrin, eine Caspase-1, ein alpha-1-Macroglobulin, ein Lysozym c-1 und eine Katalase, die allesamt in konventionellen Schaben überexprimiert waren. Die letztgenannten Gene erkennen oder bekämpfen Mikroorganismen, um das Mikrobiom zu regulieren und somit für Balance und Stabilität

innerhalb des Wirts zu sorgen. Die Regulierung von Immungenen durch das Mikrobiom könnte also dazu beitragen, dass Schaben effektiv Krankheitserreger bekämpfen können, was durch geringere Sterberaten von konventionellen Schaben im Vergleich zu mikrobenfreien Schaben nach *P. entomophila* Infektion deutlich wird.

Zusammenfassend konnte ich also zeigen, dass die Biologie des Wirts stark von mikrobiellem Leben beeinflusst wird. Die Mikroben können dabei entweder pathogener, kommensaler oder nützlicher Natur sein. Sie haben große Einfluss auf das Verhalten, die Individualentwicklung und die Physiologie des Wirts. Während pathogene Mikroorganismen den Wirt schädigen, verbessert das Mikrobiom den Stoffwechsel und die Immunabwehr des Wirts. Folglich stehen Pathogene nicht nur im Konflikt mit dem Wirt, sondern auch mit seinem Mikrobiom.

Dieser Aspekt ist erst in den letzten Jahren deutlich geworden. Fortführende Forschung ist nötig, um weitere Aspekte dieses Zusammenlebens zu entschlüsseln. Ein geeignetes Untersuchungssystem bildet dabei die von mir etablierte mikrobenfreie Schabenzuchtlinie. Wiederbesiedlungsexperimente mit einzelnen Mikrobenarten können wegweisend sein, um z.B. die spezifische Rolle von einzelnen Mitgliedern des Mikrobioms aufzuklären.

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Appendix

Supplementary Tab. 1: Ingredients of artificial diets and vitamin mix composition.

Ingredient	**Amount**
carbohydrate (sucrose)	35 % or 7 % or 21 %
protein (casein, peptone and albumin from eggs in a 3:1:1 ratio)	35 % or 7 % or 21 %
linoleic acid	0.5 %
Cholesterol	0.5 %
Wesson's salt mixture	2.4 %
Ascorbate	0.3 %
1 % agar solution	1:6 w/v
Cellulose	58 %
vitamin mix:	0.2 %
- thiamine	0.075 g
- riboflavin	0.075 g
- nicotinic acid	0.3 g
- pyridoxine	0.075 g
- folic acid	0.075 g
- meso-inositol	0.75 g
- calcium pantothenate	0.15 g
- p-aminobenzoic acid	0.075 g
- choline chloride	3.75 g
- biotin	0.003 g

Supplementary Tab. 2: GLMM post-hoc comparisons for the proportion of P and C chosen as well as Wilcox rank sum tests for the P:C ratio chosen, the P consumption, the C consumption and the total consumption.

GLMM post-hoc comparison

Comparison	**P proportion chosen**	**z**	**p**
high vs. low	0.17 vs. 0.14	-3.718	≤ 0.001
high vs. unmanipulated	0.17 vs. 0.06	-7.416	< 0.001
high vs. wounded	0.17 vs. 0.14	-2.808	0.029
low vs. unmanipulated	0.14 vs. 0.06	-2.809	0.029
low vs. wounded	0.14 vs. 0.14	0.625	> 0.1
wounded vs. unmanipulated	0.14 vs. 0.06	-3.348	0.004

Comparison	**C proportion chosen**	**z**	**p**
high vs. low	0.16 vs. 0.20	2.493	0.076
high vs. unmanipulated	0.16 vs. 0.26	5.270	< 0.001
high vs. wounded	0.16 vs. 0.19	2.245	> 0.1
low vs. unmanipulated	0.20 vs. 0.26	2.016	> 0.1
low vs. wounded	0.20 vs. 0.19	-0.148	> 0.1
wounded vs. unmanipulated	0.19 vs. 0.26	2.157	> 0.1

Supplementary Tab. 3: Statistic of the 'Bacteria growth inhibition assay'. 35 = high P diet, 7 = high C diet, 21 = B diet, B = infected with *P. entomophila*, R = wounded (Ringer-injected), N = unmanipulated.

Pairs	**Test**	**Statistic**	**df**	**p-value**
Group21N:Group21R	Group	0.94911859	1.000000	3.299438e-01
Group21N:Group21R	Time	24.45189955	1.450609	7.048421e-09
Group21N:Group21R	Group:Time	0.04940962	1.450609	9.035861e-01
Group21N:Group21B	Group	0.02082959	1.000000	8.852442e-01
Group21N:Group21B	Time	21.61958760	1.906625	9.425439e-10
Group21N:Group21B	Group:Time	0.43041662	1.906625	6.406119e-01
Group21N:Group35N	Group	0.89713864	1.000000	3.435501e-01
Group21N:Group35N	Time	32.02759629	1.692840	9.051076e-13
Group21N:Group35N	Group:Time	0.16685358	1.692840	8.105382e-01
Group21N:Group35R	Group	0.31568931	1.000000	5.742098e-01
Group21N:Group35R	Time	19.92637843	1.734186	1.943324e-08
Group21N:Group35R	Group:Time	0.75353344	1.734186	4.532869e-01
Group21N:Group35B	Group	0.08117866	1.000000	7.757064e-01
Group21N:Group35B	Time	10.43855351	1.693548	9.160941e-05
Group21N:Group35B	Group:Time	2.15346112	1.693548	1.245220e-01
Group21N:Group7N	Group	1.71132706	1.000000	1.908133e-01
Group21N:Group7N	Time	24.92417628	1.524266	2.282455e-09
Group21N:Group7N	Group:Time	0.02885406	1.524266	9.414172e-01
Group21N:Group7R	Group	1.52333935	1.000000	2.171148e-01

Group21N:Group7R	Time	12.25389405	1.375152	8.267518e-05
Group21N:Group7R	Group:Time	0.78531080	1.375152	4.128986e-01
Group21N:Group7B	Group	0.03087525	1.000000	8.605190e-01
Group21N:Group7B	Time	8.43893951	1.314370	1.501511e-03
Group21N:Group7B	Group:Time	0.50320883	1.314370	5.273906e-01
Group21N:GroupNeg	Group	16.14912642	1.000000	5.854557e-05
Group21N:GroupNeg	Time	9.38268929	1.508262	4.154548e-04
Group21N:GroupNeg	Group:Time	7.39920773	1.508262	1.951805e-03
Group21N:GroupPos	Group	10.49133738	1.000000	1.199355e-03
Group21N:GroupPos	Time	40.99995207	1.523319	9.898651e-15
Group21N:GroupPos	Group:Time	3.89836559	1.523319	3.077441e-02
Group21R:Group21B	Group	2.03135180	1.000000	1.540835e-01
Group21R:Group21B	Time	43.04350004	2.066578	5.588492e-20
Group21R:Group21B	Group:Time	0.65593248	2.066578	5.237776e-01
Group21R:Group35N	Group	0.01998132	1.000000	8.875893e-01
Group21R:Group35N	Time	74.60733002	1.674130	3.411569e-28
Group21R:Group35N	Group:Time	0.60357068	1.674130	5.184021e-01
Group21R:Group35R	Group	0.22291741	1.000000	6.368259e-01
Group21R:Group35R	Time	42.75295926	1.725827	5.248277e-17
Group21R:Group35R	Group:Time	1.26384192	1.725827	2.797789e-01
Group21R:Group35B	Group	1.79647499	1.000000	1.801392e-01
Group21R:Group35B	Time	17.68494716	1.642390	2.646545e-07
Group21R:Group35B	Group:Time	3.29732919	1.642390	4.651205e-02

Group21R:Group7N	Group	0.25611706	1.000000	6.128004e-01
Group21R:Group7N	Time	51.06667278	1.329412	4.054874e-16
Group21R:Group7N	Group:Time	0.06099755	1.329412	8.704968e-01
Group21R:Group7R	Group	0.14994576	1.000000	6.985872e-01
Group21R:Group7R	Time	19.03275846	1.206341	2.565371e-06
Group21R:Group7R	Group:Time	0.96915697	1.206341	3.407332e-01
Group21R:Group7B	Group	1.70257088	1.000000	1.919521e-01
Group21R:Group7B	Time	10.63342008	1.171614	5.732710e-04
Group21R:Group7B	Group:Time	0.49559959	1.171614	5.101771e-01
Group21R:GroupNeg	Group	66.73542052	1.000000	3.105054e-16
Group21R:GroupNeg	Time	27.94832950	1.194955	1.178499e-08
Group21R:GroupNeg	Group:Time	21.73172879	1.194955	5.318704e-07
Group21R:GroupPos	Group	13.10499661	1.000000	2.945092e-04
Group21R:GroupPos	Time	117.24217723	1.270239	6.694179e-34
Group21R:GroupPos	Group:Time	13.66198869	1.270239	5.279255e-05
Group21B:Group35N	Group	2.15224753	1.000000	1.423614e-01
Group21B:Group35N	Time	64.16508155	2.219674	1.996470e-31
Group21B:Group35N	Group:Time	1.90439619	2.219674	1.438608e-01
Group21B:Group35R	Group	0.74390471	1.000000	3.884129e-01
Group21B:Group35R	Time	37.04501081	2.946901	1.490526e-23
Group21B:Group35R	Group:Time	0.36987927	2.946901	7.711641e-01
Group21B:Group35B	Group	0.03379998	1.000000	8.541329e-01
Group21B:Group35B	Time	15.28094226	2.343061	3.001774e-08

Group21B:Group35B	Group:Time	2.12204557	2.343061	1.108029e-01
Group21B:Group7N	Group	3.23517785	1.000000	7.207248e-02
Group21B:Group7N	Time	44.07749046	2.131836	5.264760e-21
Group21B:Group7N	Group:Time	0.69893798	2.131836	5.057970e-01
Group21B:Group7R	Group	2.98936933	1.000000	8.381280e-02
Group21B:Group7R	Time	16.62138453	1.775784	2.652630e-07
Group21B:Group7R	Group:Time	0.53975830	1.775784	5.619352e-01
Group21B:Group7B	Group	0.19931718	1.000000	6.552726e-01
Group21B:Group7B	Time	9.48013158	1.468942	4.385883e-04
Group21B:Group7B	Group:Time	0.30636951	1.468942	6.672627e-01
Group21B:GroupNeg	Group	27.44778491	1.000000	1.613936e-07
Group21B:GroupNeg	Time	21.43204212	1.653764	1.067228e-08
Group21B:GroupNeg	Group:Time	16.40443172	1.653764	7.123214e-07
Group21B:GroupPos	Group	23.12578382	1.000000	1.517422e-06
Group21B:GroupPos	Time	99.10840731	1.742992	1.588133e-38
Group21B:GroupPos	Group:Time	15.06794161	1.742992	1.290688e-06
Group35N:Group35R	Group	0.16318299	1.000000	6.862428e-01
Group35N:Group35R	Time	67.12391275	2.507396	9.671261e-37
Group35N:Group35R	Group:Time	3.92319000	2.507396	1.264305e-02
Group35N:Group35B	Group	1.79459857	1.000000	1.803669e-01
Group35N:Group35B	Time	26.31603915	1.731531	7.585868e-11
Group35N:Group35B	Group:Time	6.45901587	1.731531	2.639920e-03
Group35N:Group7N	Group	0.48592858	1.000000	4.857491e-01

Group35N:Group7N	Time	76.36696465	1.962283	2.626534e-33
Group35N:Group7N	Group:Time	0.58032702	1.962283	5.565460e-01
Group35N:Group7R	Group	0.32941367	1.000000	5.660045e-01
Group35N:Group7R	Time	26.50333853	1.396735	2.873995e-09
Group35N:Group7R	Group:Time	2.43785588	1.396735	1.059601e-01
Group35N:Group7B	Group	1.99200173	1.000000	1.581317e-01
Group35N:Group7B	Time	13.72348671	1.260852	5.327106e-05
Group35N:Group7B	Group:Time	1.11315571	1.260852	3.056538e-01
Group35N:GroupNeg	Group	104.88515107	1.000000	1.294242e-24
Group35N:GroupNeg	Time	64.22514153	1.967839	3.332061e-28
Group35N:GroupNeg	Group:Time	52.02116395	1.967839	5.483420e-23
Group35N:GroupPos	Group	27.37848299	1.000000	1.672822e-07
Group35N:GroupPos	Time	213.43702302	2.232254	6.949212e-104
Group35N:GroupPos	Group:Time	18.15544068	2.232254	2.389068e-09
Group35R:Group35B	Group	0.80736405	1.000000	3.689007e-01
Group35R:Group35B	Time	13.48736509	1.857929	2.875071e-06
Group35R:Group35B	Group:Time	1.27174554	1.857929	2.791650e-01
Group35R:Group7N	Group	0.81165092	1.000000	3.676326e-01
Group35R:Group7N	Time	43.87854023	1.938901	2.936899e-19
Group35R:Group7N	Group:Time	1.38990187	1.938901	2.491491e-01
Group35R:Group7R	Group	0.64713379	1.000000	4.211393e-01
Group35R:Group7R	Time	14.89853198	1.430786	9.179687e-06
Group35R:Group7R	Group:Time	0.17027414	1.430786	7.686285e-01

Group35R:Group7B	Group	0.36251220	1.000000	5.471143e-01
Group35R:Group7B	Time	8.29997608	1.273234	1.853449e-03
Group35R:Group7B	Group:Time	0.14584603	1.273234	7.634104e-01
Group35R:GroupNeg	Group	39.69619516	1.000000	2.967030e-10
Group35R:GroupNeg	Time	21.13971402	2.189425	1.262436e-10
Group35R:GroupNeg	Group:Time	15.34769834	2.189425	6.955630e-08
Group35R:GroupPos	Group	12.57774026	1.000000	3.903684e-04
Group35R:GroupPos	Time	113.45930198	2.400727	2.078562e-59
Group35R:GroupPos	Group:Time	22.72211479	2.400727	3.038060e-12
Group35B:Group7N	Group	2.76793442	1.000000	9.617019e-02
Group35B:Group7N	Time	18.13599555	1.807859	5.416072e-08
Group35B:Group7N	Group:Time	3.51513176	1.807859	3.419270e-02
Group35B:Group7R	Group	2.54787656	1.000000	1.104426e-01
Group35B:Group7R	Time	6.54410078	1.421472	4.538533e-03
Group35B:Group7R	Group:Time	0.35454595	1.421472	6.276811e-01
Group35B:Group7B	Group	0.30580929	1.000000	5.802632e-01
Group35B:Group7B	Time	4.32182269	1.325524	2.687507e-02
Group35B:Group7B	Group:Time	0.41991021	1.325524	5.735113e-01
Group35B:GroupNeg	Group	13.75216533	1.000000	2.085802e-04
Group35B:GroupNeg	Time	3.28903762	1.542975	4.989698e-02
Group35B:GroupNeg	Group:Time	1.85681020	1.542975	1.656239e-01
Group35B:GroupPos	Group	13.79132907	1.000000	2.042769e-04
Group35B:GroupPos	Time	39.29126513	1.661308	3.262590e-15

Group35B:GroupPos	Group:Time	15.89438419	1.661308	1.041167e-06
Group7N:Group7R	Group	0.01641870	1.000000	8.980418e-01
Group7N:Group7R	Time	19.44686602	1.315940	8.259811e-07
Group7N:Group7R	Group:Time	1.08202367	1.315940	3.162081e-01
Group7N:Group7B	Group	3.05774137	1.000000	8.035329e-02
Group7N:Group7B	Time	10.78001076	1.231912	4.133871e-04
Group7N:Group7B	Group:Time	0.59518304	1.231912	4.734470e-01
Group7N:GroupNeg	Group	62.03685410	1.000000	3.370892e-15
Group7N:GroupNeg	Time	29.19420640	1.333420	9.517116e-10
Group7N:GroupNeg	Group:Time	22.83049110	1.333420	7.159171e-08
Group7N:GroupPos	Group	6.16661099	1.000000	1.301838e-02
Group7N:GroupPos	Time	121.85368479	1.409850	1.028649e-38
Group7N:GroupPos	Group:Time	12.29056253	1.409850	6.865922e-05
Group7R:Group7B	Group	2.80758093	1.000000	9.381975e-02
Group7R:Group7B	Time	5.60391293	1.178670	1.351174e-02
Group7R:Group7B	Group:Time	0.11007043	1.178670	7.817342e-01
Group7R:GroupNeg	Group	66.36295411	1.000000	3.750857e-16
Group7R:GroupNeg	Time	4.93246497	1.180684	2.087925e-02
Group7R:GroupNeg	Group:Time	3.34895134	1.180684	6.006671e-02
Group7R:GroupPos	Group	8.02597902	1.000000	4.611109e-03
Group7R:GroupPos	Time	35.82584734	1.221539	6.342823e-11
Group7R:GroupPos	Group:Time	8.80331805	1.221539	1.542777e-03
Group7B:GroupNeg	Group	72.34710123	1.000000	1.804877e-17

Group7B:GroupNeg	Time	2.72490245	1.157154	9.315275e-02
Group7B:GroupNeg	Group:Time	1.95530023	1.157154	1.599135e-01
Group7B:GroupPos	Group	50.07894902	1.000000	1.476829e-12
Group7B:GroupPos	Time	15.98683761	1.178351	2.050914e-05
Group7B:GroupPos	Group:Time	4.65137016	1.178351	2.519028e-02
GroupNeg:GroupPos	Group	570.68751382	1.000000	3.978910e-126
GroupNeg:GroupPos	Time	365.20325224	1.442484	6.750337e-116
GroupNeg:GroupPos	Group:Time	317.55776029	1.442484	5.891786e-101

Supplementary Tab. 4: List of the statistically significant abundant (> 2-fold more) hemolymph proteins with their function of *B. orientalis* males after immune challenge with 5.8 x 10^5 *P. entomophila* CFUs and assigning them to a P-rich or C-rich diet.

Name	Main biological process	Gene ID	Blast e-value	Reference (see **Bibliography**)
Hexokinase type II	Carbohydrate metabolism (glycolysis)	HXK2_DROME	2.81e-176	Yanagawa 1978
Carbonyl reductase III	NADPH-dependent reduction of biologically and pharmacologically active substrates including endogenous and xenobiotic carbonyl compounds	CBR3_MOUSE	3.59e-84	Hoffmann and Maser 2007
L-galactose dehydrogenase	Unkown	GALDH_ARATH	2.77e-75	
Tropomyosin	Calcium dependent regulation of muscle contraction	TPM_PERAM	0.0	Pomés *etal.* 2007
Acyl-CoA-binding protein	Transport (Lipid binding); Suppression of glucose-induced insulin secretion	ACBP_CHICK	7.22e-33	Færgeman *etal.* 2007; Pasco and Léopold 2012
Alpha-amylase	Carbohydrate metabolism	AMY_TENMO	2.75e-89	Terra and Ferreira 1994
Proteasome subunit alpha type-III	Protein degradation	PSA3_MOUSE	2.4e-131	Rivett 1993
Hemolymph lipopolysaccharide-binding protein-like (2 isoforms)	Carbohydrate binding (probably foreign particles)	LPSBP_PERAM	6.6e-48 5.83e-64	Jomori and Natori 1991
Superoxide dismutase	Extracellular superoxide metabolic process	SODE_CAEEL	1.13e-47	Felton and Summers 1995
Ankyrin-1	Unkown	ANK1_MOUSE	7.01e-38	
Glutamine synthetase	Glutamate catabolic process; Glutamine biosynthetic process; Neurotransmitter receptor metabolic process	GLNA2_DROME	0.0	Smartt *etal.* 1998
Adenylate kinase isoenzyme I	ATP metabolic process	KAD1_PIG	3.31e-46	Fujisawa *etal.* 2009
Hexamerin	Nutrient reservoir activity (amino acid and energy storage)	HEXA_BLADI	1.77e-83	Burmester 1999

BeGenDiv protocol

Dual indexing

- Quantitation DNA (use a dsDNA binding dye => Qubit, PicoGreen plate fluorometer
- Ideally use the same amount of DNA for each sample

1. First PCR (target specific PCR)

PCR master mix

16,1	µl H2O
5	µl Buffer
1	µl Forward Primer (10pm/µl)
1	µl Reverse Primer (10pm/µl)
0,5	µl dNTP (each 25mM)
0,4	µl Herculase II Fusion DNA Polymerase
x	µl BSA (10mg/ml)
x	µl DMSO
x	µl MgCl2
1	µl solution of DNA template
25	µl Total

Template depending, adapt H_2O accordingly

PCR program

95°C	2 min
30	**Cycles***
95°C	30s
x	30s
72°C	30s
72°C	x
4°C	hold

red: depends on primer and product

*Number of cycles should not exceed 30

2. Purification first PCR with magnetic beads

1. vortex High Prep PCR beads thoroughly
2. add beads in a 0,8: 1,0 (beads: PCR) ratio to the PCR reaction, mix
3. incubate at room temperature for 5min (without shaking)
4. place tube/ plate on magnetic rack for 2-5min
5. keep tubes/ plate on magnetic rack and remove supernatant carefully without spoiling the bead pellet (make sure you have NO beads in the pipet)
6. add 200µl 70% EtOH (freshly prepared)
7. incubate 10s
8. remove supernatant

9. add 200µl 70% EtOH a second time

10. incubate 10s

11. remove supernatant

12. air dry pellet or incubate at 37°C for app. 10 minutes (make sure not to overdry the beads, open lids of tubes!)

13. add 25 µl 1x TE

14. remove tubes/ plate from magnetic rack

15. vortex gently

16. incubate 5min at room temperature

17. place tube on magnetic rack for 2min

18. transfer 24 µl of the supernatant to a fresh tube/ plate

19. Quantitation DNA (use a dsDNA binding dye => Qubit, PicoGreen plate fluorometer

20. Use the same amount of DNA for each sample to do the subsequent PCR

3. Second PCR (indexing PCR)

PCR master mix

7,25	µl H2O
5	µl Buffer
0,625	µl Index P7_X
0,625	µl Index P5_X
0,25	µl dNTP (each 25mM)
1	µl DMSO
0,25	µl Herculase II Fusion DNA Polymerase
10	µl solution of PCR product
25	µl Total

PCR program

95°C	2 min
8	**cycles**
95°C	20s
52°C	30s
72°C	30s
72°C	3 min

Each sample gets an individual index combination!

4. Purification second PCR with magnetic beads, step A

1. vortex High Prep PCR beads thoroughly

2. add beads in a 0,8: 1,0 (beads: PCR) ratio to the PCR reaction, mix

3. incubate at room temperature for 5min (without shaking)

4. place tube on magnetic rack for 2-5min

5. keep tubes/ plate on magnetic rack and remove supernatant carefully without spoiling the bead pellet (make sure you have NO beads in the pipette)

6. add 200µl 70% EtOH (freshly prepared)

7. incubate 10s

8. remove supernatant

9. add 200µl 70% EtOH a second time

10. incubate 10s

11. remove supernatant

12. air dry pellet or incubate at 37°C for app. 10 minutes (make sure not to overdry the beads, open lids of tubes!)

13. add 25 µl 1x TE

14. remove tubes/ plate from magnetic rack

15. vortex gently

16. incubate 5min at room temperature

17. place tube on magnetic rack for 2min

18. transfer 24 µl supernatant to a fresh tube

5. Purification second PCR with magnetic beads, step B

1. vortex High Prep PCR beads thoroughly

2. add beads in a 0,8: 1,0 (beads: PCR) ratio to the PCR reaction, mix

3. incubate at room temperature for 5min (without shaking)

4. place tube on magnetic rack for 2-5min

5. keep tubes/ plate on magnetic rack and remove supernatant carefully without spoiling the bead pellet (make sure you have NO beads in the pipette)

6. add 200µl 70% EtOH (freshly prepared)

7. incubate 10s

8. remove supernatant

9. add 200µl 70% EtOH a second time

10. incubate 10s

11. remove supernatant

12. air dry pellet or incubate at 37°C for app. 10 minutes (make sure not to overdry the beads, open lids of tubes!)

13. add 21 µl 1x TE

14. remove tubes/ plate from magnetic rack

15. vortex gently

16. incubate 5min at room temperature

17. place tube on magnetic rack for 2min

18. transfer 20 µl supernatant to a fresh tube

6. Check indexing PCR and pooling

1. check ten samples of the index PCR and one sample of target PCR on Agilent:
Do you see a clear length shift between both PCRs? It is normal to see three fragment peaks after the indexing PCR. Usually the indexing doesn't work perfectly.
2. Quantitation indexing PCRs with PicoGreen in duplicates or qPCR (depends on budget, qPCR detects only fragment with complete Illumina adaptors, PicoGreen detects the whole dsDNA)
3. Pool samples (in equimolar) ratio