MARSHALL PLAN SCHOLARSHIP PAPER

INFLUENCE OF YOUNG AGE MICROBIOME ON ADULT SLEEP BEHAVIOR IN *D.MELANOGASTER*

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1. Abstract

There is growing evidence for the interaction between gut microbiome and the brain. Several studies reported correlations between the composition of gut microbiome and various neurological diseases. Moreover gut bacteria are shown to influence levels of neurotransmitters such as GABA, which are unbalanced in stress related disorders, such as anxiety and depression and also in in sleep disorders.

Drosophila Melanogaster is a powerful model organism for investigating the mechanisms of gut microbiome-brain link. In addition to a plethora of available genetic techniques, yielding germ free (axenic) flies and establishing gnotobiotic cultures is much faster and easier with fruit flies compared to other model organisms. Moreover *Drosophila* microbiome is much simpler in complexity in contrast to vertebrates.

Fruit flies are extensively studied in sleep research, but sleep and gut bacteria relationship hasn't been studied yet. During my project I investigated the significance of early adulthood microbiome on adult life sleep in *Drosophila*. My hypothesis was that differences in microbiome composition might elucidate the reason for the behavioral variability in resilience/vulnerability to sleep deprivation, between individuals with same genetic background.

2. Introduction

Why do we sleep? What is the function of sleep? Sleep remains to be one of the great mysteries of neuroscience research. Why would a highly vulnerable "offline" state be evolutionarily preferable? Over the years a great number of studies addressed these questions, nonetheless the ultimate function of sleep still remains unknown. Is it for energy conservation? Is it important for development? Is it to strengthen the synapses? Or is it just to erase useless memories? Why did sleep evolve? One convincing theory is that sleep evolved to keep up with the increasing complexity of the nervous systems. As brains got more complex, so did the tasks and the required physiological processes to achieve those tasks. Sleep served as a function to manage the increasing complexity. But what does sleep change in the brain? Is it a change in synapse number? Or does sleep reshape the neural connections?

2.1. Function of sleep: What is known so far?

In our modern world sleep disturbances and its subsequent consequences affect a great number of people. The National Institute of Health (NIH) reports show that almost 40 million Americans suffer from chronic long-term sleep disorders and an additional 20 million experience occasional sleeping problems (numbers are taken from NIH- Neurological disorders and stroke website).

Numerous studies aimed to pin down the function of sleep to a single purpose. Yet after almost 200 years of research, the field doesn't have a consensus on a one definite theory. It is undeniable that sleep loss has profound negative effects on health. One obvious consequence of a bad night sleep, we all encounter is feeling inattentive the next day.

Chronic sleep deprivation can be the underlying cause or the consequence of other health conditions. A huge spectrum of diseases have been associated with sleep problems; few examples to name are ADHD (Attention deficit hyperactivity disorder) (reviewed in Cohen-Zion & Ancoli-Israel 2004) , Alzheimer's disease (reviewed in Ju et al. 2014) and depression (reviewed in Nutt et al. 2008). Therefore sleep researchers aim to understand what sleep does to the brain and how lack of it "breaks" a healthy brain in various ways.

However one can also look at the problem from another point of view and ask whether sleep can be used as a treatment to fix “broken” brains.

Sleep is universal to all mammals, however sleep duration varies massively between animals (Campbell & Tobler 1984) (Table 1). For example giraffes (*Giraffa camelopardalis*) are reported to have the shortest sleep time among mammals (Tobler & Schwierin 1996). Whereas Koala bears (*Phascolarctos cinereus*) spend almost 90% of the day sleeping (Ellis et al. 1995; Ryan et al. 2013).

	Average sleep in hours
Koala	20 hrs
Tiger	15.8 hrs
Cat	12.1 hrs
Chimpanzee	9.7 hrs
Sheep	3.8 hrs
African elephant	3.3 hrs
Giraffe	1.9 hr

Table 1. Sleep time duration across animals (Campbell & Tobler 1984)

Sleep or sleep like states have been reported in most of the animal species (Campbell & Tobler 1984). Currently the simplest animal with a sleep like state is considered to be *C. elegans* (Raizen et al. 2008; Trojanowski & Raizen 2015). *C. elegans* shows two sleep like states; DTS (developmentally timed sleep) and SIS (Stress induced sleep), DTS occurs during a transition state during development, called as lethargus and sleep at this state is functionally similar to sleep in insects and mammals (A. J. Hill et al. 2014). SIS is stage independent and occurs in response to environmental stressors, such as heat, cold (A. J. Hill et al. 2014).

So when and why did sleep become a necessity for survival? One compelling theory comes to mind if you look at the evolution of sleep across animal kingdom (Kirszenblat & van Swinderen 2015) (Figure 1.) Jellyfish (*Aurelia aurita*) shows a basic amount of neural complexity and are able to perform simple operant learning tasks (Johnson & Wuensch 1994), however no sleep or sleep like state have been observed in these species so far. As mentioned

before *C. elegans* is the simplest animal with a sleep like state and shows habituation and simple associative learning (Ardiel & Rankin 2010). Yet so far adult *C. elegans* sleep hasn't been shown to be critical for survival under normal conditions or to maintain the learning capabilities. On the contrary sleep appears to be critical for animals, which are capable of operant learning, including simpler invertebrates such as mollusks. Several mollusk species, especially sea hare (*Aplysia californica*) have been proven to be very useful model systems for understanding the mechanisms of memory formation and neuronal plasticity (Walters et al. 1979; Hawkins et al. 1983; Kandel 2001; Hawkins et al. 2006). Sleep in mollusks has been reported in earlier studies, however its detailed characterization has been published just recently (Vorster et al. 2014). A particularly important invertebrate model system for sleep is *Drosophila* (Shaw et al. 2000; Hendricks, Finn, et al. 2000). Sleep studies in flies have been very advantageous, particularly for demonstrating the essential role of sleep in learning and memory (Graves et al. 2001). In conclusion, considering the evolution of sleep with regard to increasing brain complexity suggests that sleep states evolved primarily as a mechanism to cope with environmental stress, but later on as a mechanism to support the physiological needs for complex learning capabilities.

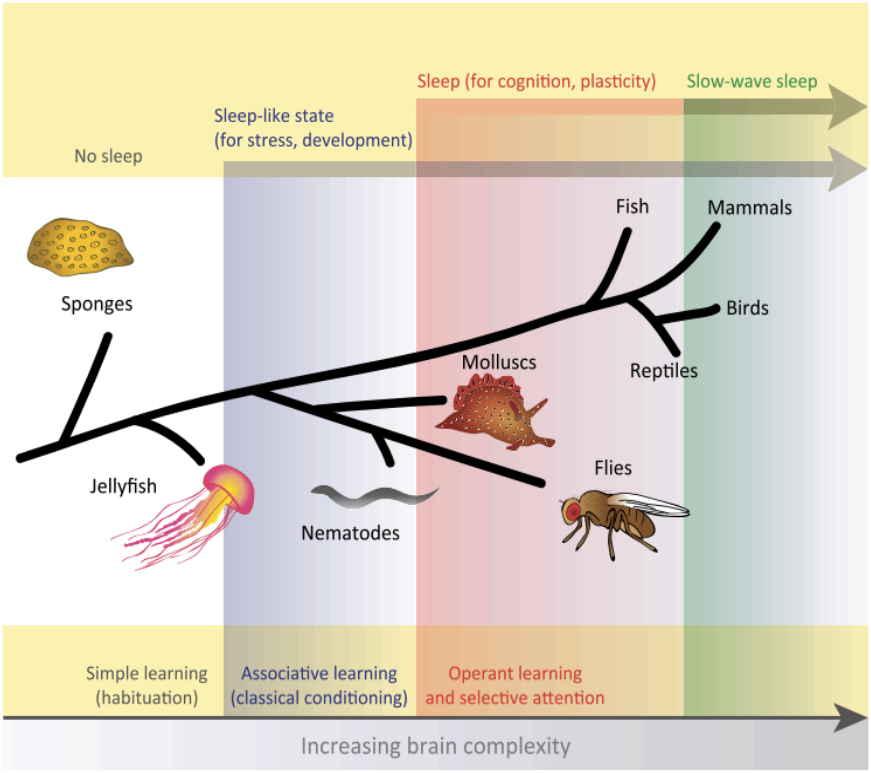

Figure 1. The evolution of sleep across animal kingdom (adapted from Kirszenblat & van Swinderen, 2015)

2.1.1. Sleep studies in humans

Twin studies in the early 1930s gave the first indications for the genetic control of sleep (Geyer 1930). The invention of electroencephalogram (EEG) revolutionized the sleep research in humans (Berger 1929). EEG recordings during wake and sleep revealed characteristic changes in brain activity (Loomis et al. 1936). A typical pattern of an EEG recording, consisting of wake, REM and NREM is shown in Figure 2. Sleep onset is usually associated with a slowing of EEG activity and a typical sleep pattern consists of one to three nonrapid eye movement stages (NREM) and a REM stage. NREM and REM sleep alternate throughout the night. One entire cycle is approximately 90 minutes and the cycle is repeated several times throughout the night (Figure 2A).

Sleep stages revealed characteristic EEG traces. The waking EEG shows a low voltage fast activity in the beta (>13Hz) and alpha (8-13Hz). REM sleep also called as the paradoxical sleep revealed high cortex activity in EEG recordings with a theta activity. The three stages of NREM have different EEG characteristics. N1 is a transitional stage with mixed voltage activity. At N2 stage alpha activity disappears, and 12-14Hz sinusoidal waves called sleep spindles can be seen. Slow wave sleep (SWS), occurs during stage 3 of NREM and is the deepest stage of sleep. (Cirelli 2009; Diekelmann & Born 2010) (Figure 2B).

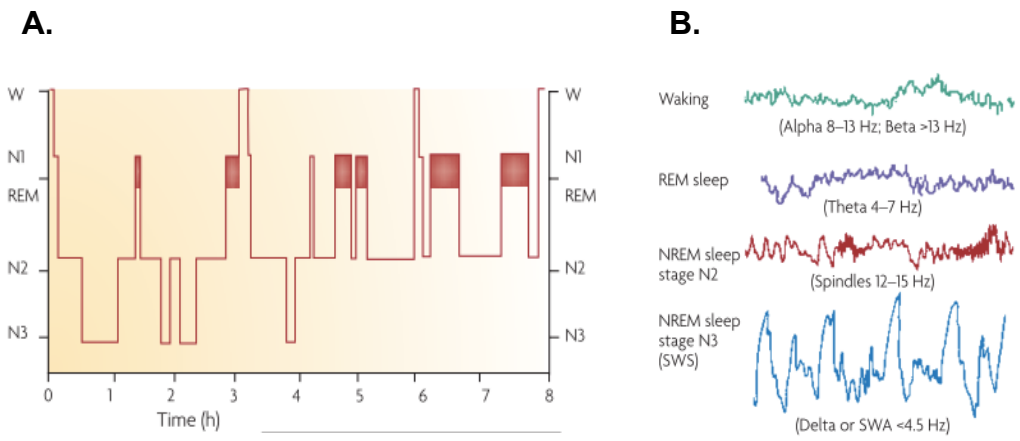

Figure 2. Representative electroencephalogram (EEG) traces (adapted from Cirelli 2009)
A.Distribution of sleep stages in adult humans
B. EEG traces of waking, REM and NREM stages

2.1.2. Conceptual models of sleep-wake regulation

- The two-process model and synaptic homeostasis hypothesis

The two-process model describes the main forces that control sleep and wake cycle, namely the 24h circadian rhythm and homeostatic processes (Figure 3.). Process S stands for sleep propensity and it increases during waking and decreases during sleep. Process C represents the circadian rhythm and sets thresholds for process S. According to this model when process S reaches the upper threshold of process C during waking, sleep should occur. And when process S decreases to lower circadian threshold waking occurs. This model suggests that under normal conditions synaptic potentiation increases during wake periods and peaks before switching to a sleep state upon which synaptic downscaling returns back to baseline.

The synaptic homeostasis hypothesis (SHY) is an extension of the two-process model and brings plasticity in to the picture (Figure 4). Brain plasticity is essential to learn and adapt to a changing environment. SHY suggests a potentiation in synapses during wakefulness as a result of learning. Sleep is a process to downscale the synapses and thus restore the cellular levels back to baseline, which are required for optimum learning during wakefulness. SHY associates slow-wave activity with the synaptic changes, as it correlates with the homeostatic regulation of sleep need(Tononi & Cirelli 2006; Tononi & Cirelli 2014).

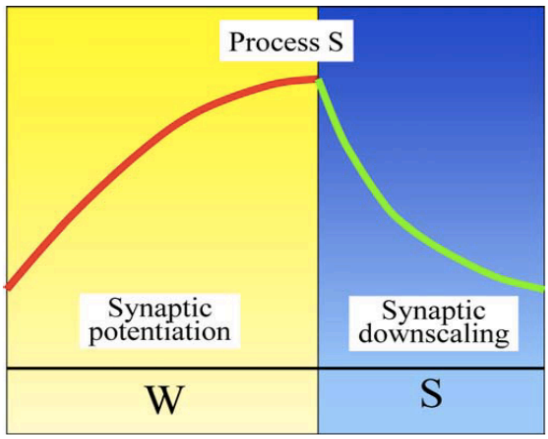

Figure 3. The two-process model (adapted from Tononi & Cirelli, 2006)
Process S stands for sleep propensity, which increases during wake and reaches a maximum before sleep, which is defined by process C, the circadian component of the model.

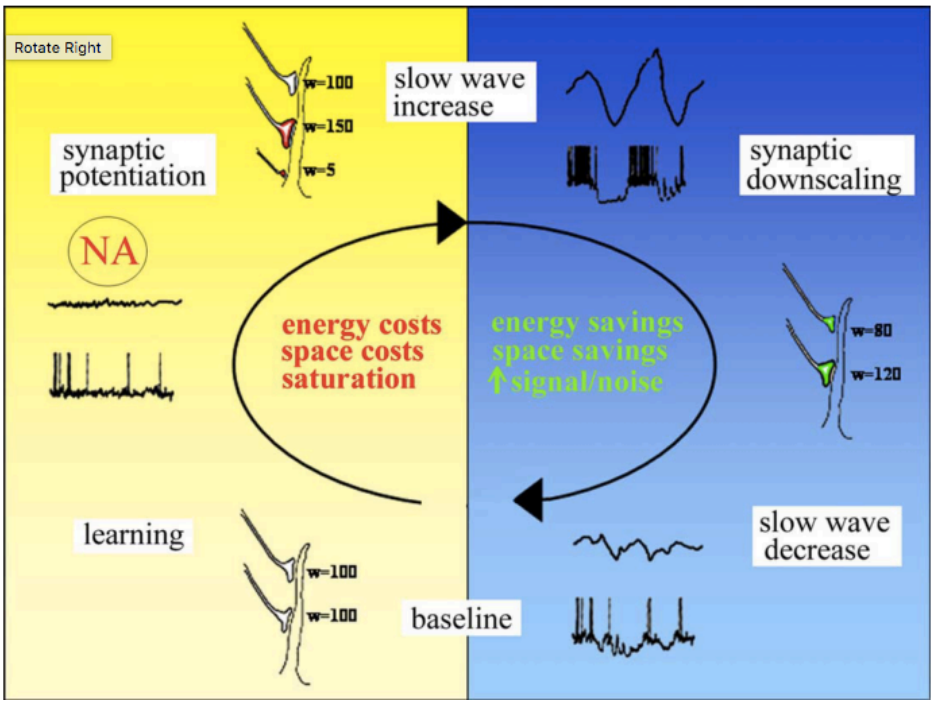

Figure 3. Synaptic homeostasis hypothesis (adapted from Tononi & Cirelli, 2006)
According to SHY one of the functions of sleep is a synaptic downscaling. Synaptic potentiation which occurs during learnig through wakefullnes, ensures the plastic changes in the brain. However plasticity has a high cellular cost, both regarding the energy and the space. Slow- wave decrease during sleep ensures that the cellular mechanism return to a baseline before wake. This homeostatic balance is essential for the optimal functining of the brain.

2.1.3. Sleep research in flies

The genetic toolbox of simpler model systems such as *C. elegans*, *Drosophila Melanogaster* and *Danio rerio* led to a rapid progress in sleep research (Allada & Siegel 2008; Cirelli 2009). The fruit fly, *Drosophila Melanogaster* became a particularly attractive model for sleep research.

Drosophila Melanogaster as a model system

Introduced by Thomas Hunt Morgan 100 years ago, fruit fly is one of the best-studied model systems and contributed greatly to various aspects of biology. Fruit fly offers several advantages as a model system. A short life cycle, ease of culture and maintenance and small genome size makes are few practical features, which make *Drosophila* a great model system. Additionally

Drosophila offers a plethora of genetic techniques, such as binary expression systems (Brand & Perrimon 1993), optogenetics (Inagaki et al. 2013), CRISPR/Cas9 (Bassett & Liu 2014), which allow researchers a variety of sophisticated manipulations. The *Drosophila* genome consists of approximately 16.000 genes, with four pairs of chromosomes (Hild et al. 2003). *Drosophila* shows almost 75% overlap with human disease genes and therefore has been instrumental towards better understanding of human diseases (Reiter et al. 2001).

Neuroscience is probably the scientific field that profited the most from fly research. A combination of genetics and the possibility to monitor behavior relatively easily paved the way for fly neuroscience. The fruit fly not only provided a great understanding to the fundamental features of the nervous system organization and function, but it also served as a tool to study the genes involved in neurological diseases in much greater detail.

Sleep in flies was first described 16 years ago (Shaw et al. 2000; Hendricks, Sehgal, et al. 2000; Hendricks, Finn, et al. 2000). These findings opened up a new era for sleep research and led to identification of molecular mechanisms of sleep in much greater detail. Since then a number of genes and neural pathways involved in sleep regulation, have been identified by utilizing fly as a model system. These studies revealed that flies met the criteria for sleep as it was previously described in mammals, which are defined as (1) periods of quiescence with increased arousal thresholds, (2) rapid reversibility and (3) homeostasis (Campbell & Tobler 1984). (1) Flies showed extended durations of immobility, independent of light conditions. These immobile periods last approximately 9-10 hours per day in adult flies. The observation of rest periods in a circadian mutant, timeless, further supported that this inactivity is not a simple circadian clock observation (Hendricks, Finn, et al. 2000). (2) These rest states were rapidly reversible and demonstrated increased arousal thresholds, similar to the characteristics of mammalian sleep. Another convincing evidence was the presence of a homeostatic sleep response (3). When flies were deprived of sleep, they made up for the lost sleep on the following day. Later on, supplementary evidence came from electrophysiological studies, where frequency of oscillations of EEG-like local

field potentials (LFPs) showed behavioral state dependent electrical activity (Nitz et al. 2002).

The possibility to conduct forward genetic screens by using high- throughput assays, allowed identification of novel genes involved in fruit fly sleep. *Minisleep* (*mms*) was identified in an EMS (ethyl methane sulfonate) screen and had a significant decrease in sleep amount (Cirelli et al. 2005). These flies had shorter sleep episodes and recovered their sleep similar to wild type flies when deprived. Interestingly *mns* flies showed no behavioral impairment upon sleep deprivation. Further investigations revealed that *mns* mutation was a point mutation in *Shaker*, a gene that encodes the alpha subunit of a tetrameric potassium channel. As expected *Shaker* mutants also showed reduced sleep phenotype. Later on in another EMS screen *insomniac* (*inc*) mutant was identified (Stavropoulos & Young 2011). *Inc* mutants also had a drastic reduction in their total sleep time. *Inc* was shown to be encoding for a putative adapter, binding to Cullin-3 ubiquitin ligase complex, which is involved in sleep regulation. Another mutant was identified in a RNA interference (RNAI) screen, *Cyclin A* (*Rca1*) (Rogulja & Young 2012). Pan-neuronal *Rca1* depletion using elav-Gal4 led to a decrease in sleep levels. *Rca1* is however also expressed in clock neurons, which are involved in circadian regulation of sleep.

Another interesting sleep mutant is *sleepless* (sss) (Koh et al. 2008). The sleepless gene is shown to be encoding for a brain-enriched, glycosylphosphatidylinositol-anchored protein and loss of sleepless resulted in a drastic 80% reduction in sleep (Figure 4.).

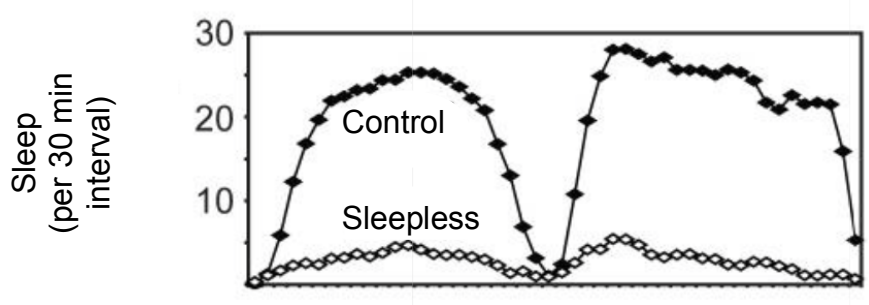

Figure. 4 Sleep profiles of control and sleepless flies (adapted from (Koh et al. 2008)
Sleepless mutants show a drastic reduction in both daytime and nighttime sleep

But how do these genes affect the physiological mechanisms, which regulate sleep. One physiological important function of sleep is the regulation of synaptic plasticity (Donlea Ramanan, N., Shaw, P. J. et al. 2009; Donlea et al. 2011; Vanderheyden et al. 2013; Tononi & Cirelli 2014). If you look at the SHY model of sleep, the need for sleep becomes evident upon reaching a threshold, which suggests a saturation of synapses. Sleep serves as a mechanism to bring the synapses back to a cellular baseline. First structural indices for SHY came from social enrichment studies in *Drosophila*. Social enrichment is shown to be inducing plasticity in circuits throughout the brain(Volkmar & Greenough 1972; Technau 1984). As expected flies, which were raised in groups slept significantly longer than isolated flies. These changes were however not observed in classic memory mutants, such as *rutabaga* and *dunce* (Ganguly-Fitzgerald et al. 2006).

Another support for SHY was the finding, which showed an increase in protein

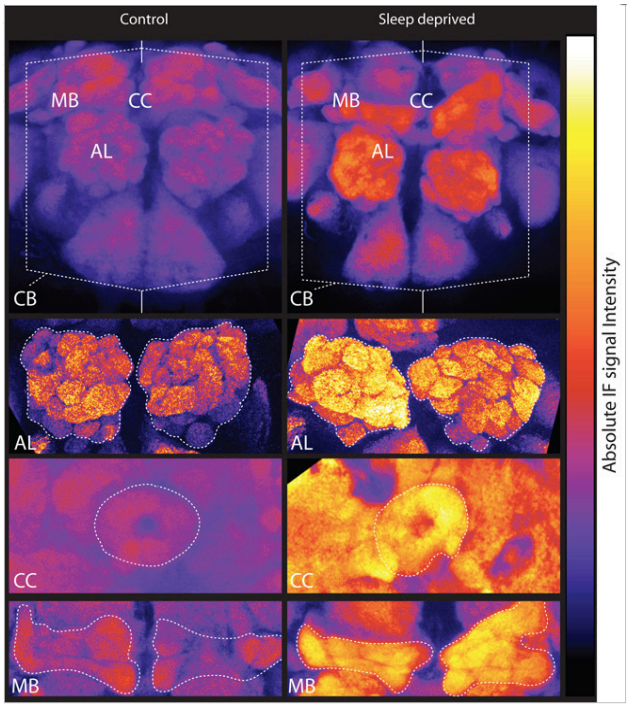

Figure 5. Bruchpilot (BRP) immunofluorescence in controls and 16h sleep deprived flies (Gilestro et al. 2009)

Immunoreactivity levels measured in antennal lobes (AL), beta lobes of the mushroom bodies (MB), ellipsoid body of the central complex (CC) and central cerebrum (excluding the optic lobes, CB).

levels of pre- and post synaptic markers; such as Bruchpilot, synapsin, syntaxin after wake and their decrease after sleep (Gilestro et al. 2009).
Further support for the role of sleep in plasticity came from imaging studies of Lnv-neurons (Donlea Ramanan, N., Shaw, P. J. et al. 2009). LNv projections were indeed reduced after sleep and as predicted from SHY sleep deprivation prevented this reduction. Later on several mice studies revealed similar results providing further evidence the SHY hypothesis (Maret et al. 2011; Frank 2012).

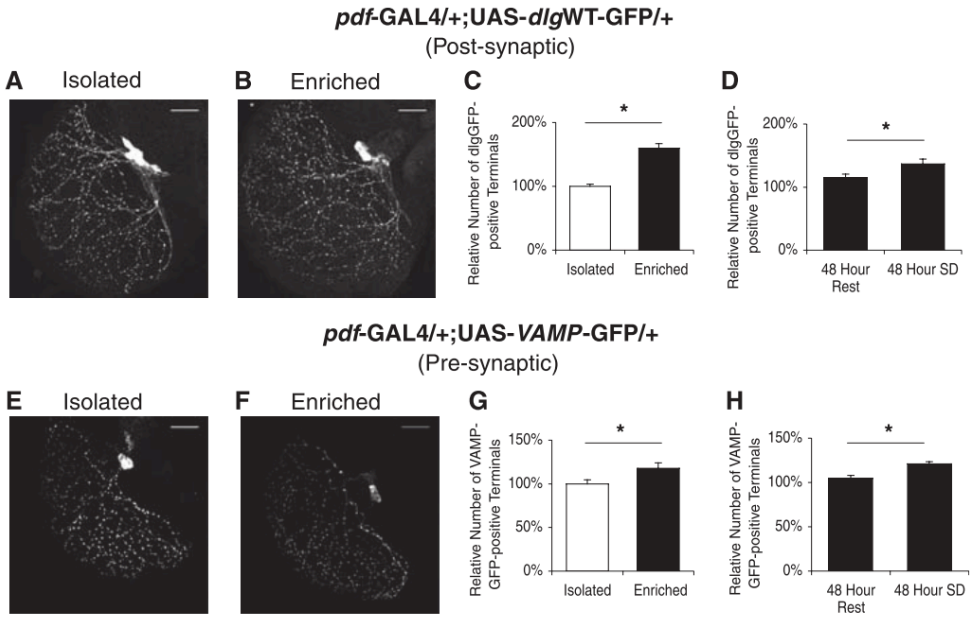

Figure 6. Social experience alters synaptic terminal numbers in LNv-neurons (adapted from Donlea et al. 2009)

Social experiences that induce increased sleep shows a significant increase in the number of synaptic terminals in the LN_V projections into the medulla (post-synaptic A,B and C),(Pre-synaptic E,F and G). The numbers of synaptic terminals were reduced after rest, however sleep deprivation prevented this decline.

Drosophila brain areas involved in sleep wake regulation

The analyses of neural circuits associated with sleep were relatively easy to identify in flies. The availability of driver lines that allow cell specific gene

expression provided a convenient tool to functionally dissect and identify neurons involved in sleep regulation.

So far six distinct brain areas involved in sleep-wake regulation have been identified (Figure 6); Mushroom Bodies (MB) (Pitman et al. 2006; Joiner et al. 2006), Ventral lateral neurons (LNv) (Parisky et al. 2008; Sheeba et al. 2008), pars intercerebralis (PI) (Crocker et al. 2010; Foltenyi et al. 2007), dorsal fan-shaped body (Donlea et al. 2011), octopamine expressing neurons (Crocker et al. 2010; Foltenyi et al. 2007) and dopamine expressing neurons (Liu et al. 2012; Ueno, Tomita, Kume, et al. 2012)(reviewed in (Dissel & Shaw 2013).

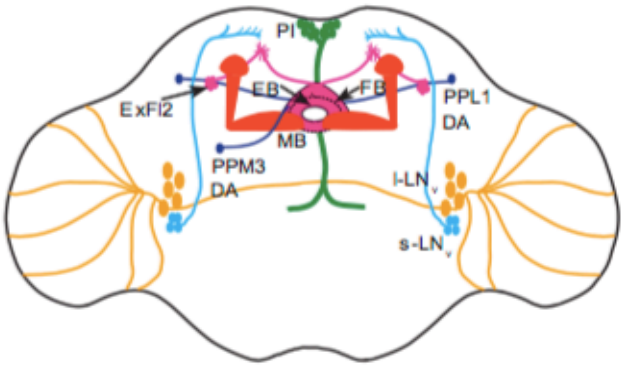

Figure 7. Drosophila brain areas involved in sleep-wake regulation
(image adapted from (Potdar & Sheeba 2013)
Mushroom bodies (MB), (Red)
L-LNvs (orange)
s-LNvs(light blue)
EB andFB (pink)
Pars Intercerebralis (green)
Dopaminergic neurons (dark blue)

MBs are higher brain centers of the fly brain involved in the regulation of various behaviors, particularly important for learning and memory(Heisenberg et al. 1985; Solanki et al. 2015; McGuire et al. 2001). MB has been also implicated in the control of sleep and wake (Pitman et al. 2006; Haynes et al. 2015; Sitaraman et al. 2015; Laurent Seugnet, Yasuko Suzuki, Lucy Vine, Laura Gottschalk 2008; Joiner et al. 2006). MB micro-circuitry, which regulates sleep is highly complex, a recent study used the split gal4 technique in combination with thermogenetic TrpA1 activation to identify the specific MB

neurons that control sleep(Sitaraman et al. 2015). While previous studies reported dopaminergic (DA) neurons in the PPL1 and PPM3 as wake promoting neurons (Ueno, Tomita, Tanimoto, et al. 2012), this study identified a new set of dopaminergic neurons, which innervate MB.

2.1.4 Importance of early age sleep

Early age is a fragile stage for development, especially for the developing brain. Mammals display enhanced sleep during early life, which is considered to be essential for cortical development and brain plasticity (Halbower et al. 2006; Ednick et al. 2009). Similar to what is observed in mammals, young flies also sleep longer then adults. This prolonged early age sleep was shown to be vital for a normal brain development and adult behavior (Murakami & Keene 2014; Kayser et al. 2014; Seugnet et al. 2011). Flies, which were sleep deprived during early ages presented learning deficits as adults.

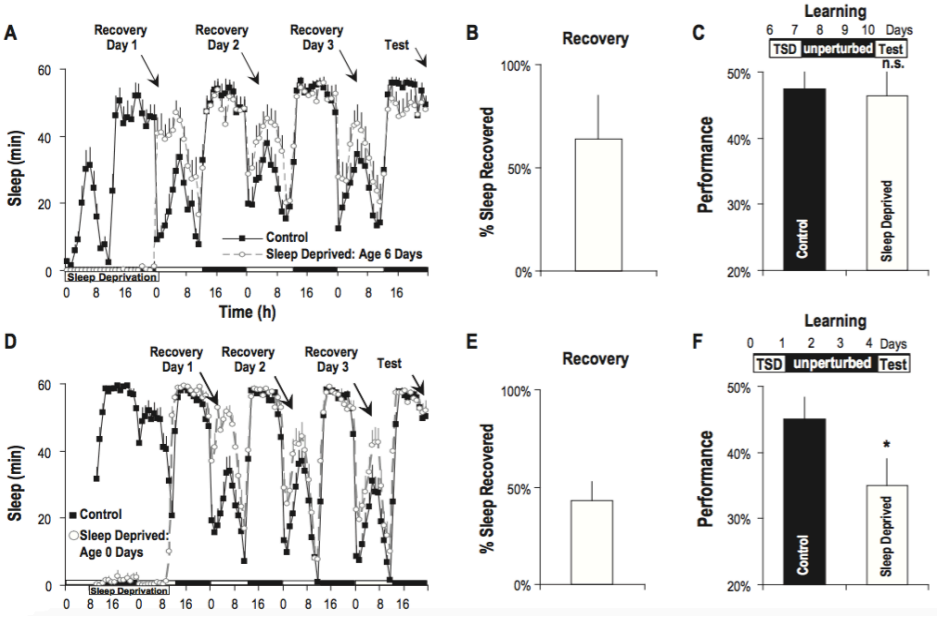

Figure 8. Sleep Deprivation During Early-Adult Development Results in Long-Lasting Learning Deficits in Adult Drosophila
(Seugnet et al. 2011)
24 h Sleep deprivation at 6 days of age (A) and 0 days (D), flies were then allowed to recover for 3 days, (B)(E) %sleep recovery compared to sleep deprived adults was not altered. (C)(F) Performance in the APS was not affected when flies where sleep deprived as adults but was significantly impaired with flies that were sleep deprived at young age.

Interestingly, these deficits were rescued by blocking the dDA1 receptor, suggesting the involvement of dopamine signaling. A following study identified that these were indeed regulated by a set of dopaminergic neurons, which project to dorsal fan shaped body (dFSB); one of the main sleep regulatory centers in the fly brain. These neurons are less active during young age and allow dFSB to be more active and promote sleep. Whereas adult flies show an increase in DA neuron activity, which then suppress the dFSB activity, leading to a decrease in sleep. In addition to the irreversible brain abnormalities, same study reported behavioral consequences upon early age sleep loss. They found that early age sleep loss led to deficits in courtship behavior.

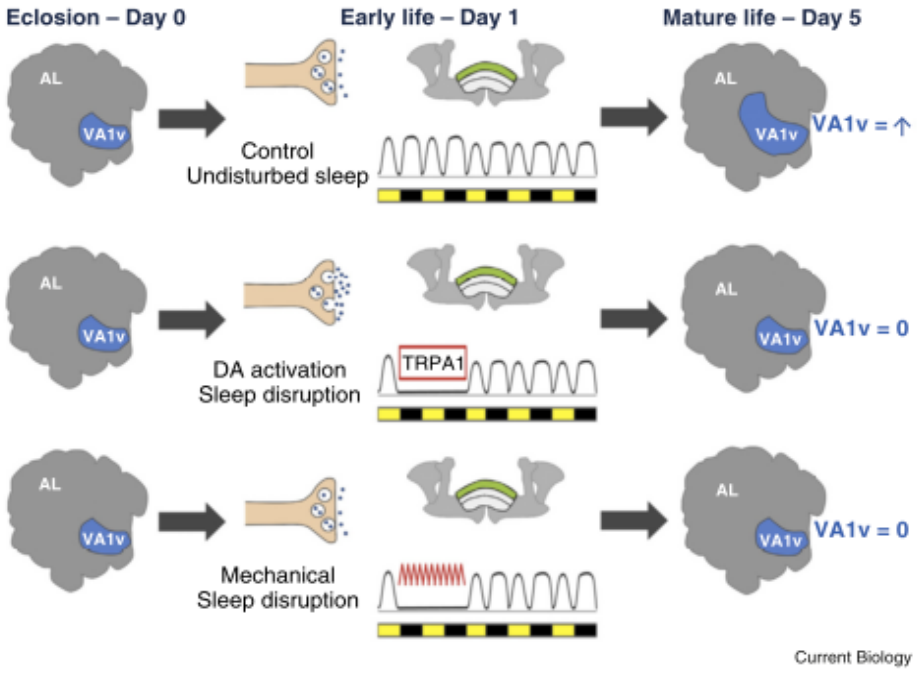

Figure 9. Dopamine-dependent modulation of VA1v development.
(figure adapted from Murakami & Keene 2014)
VA1 glomerulus of the antennal lobe continues its development during early life. (A) control flies sleep is undisturbed, therefore the dopaminergic signaling to dFSB Is reduced and allows the proper development of VA1v. (B) and (C) depicts sleep deprived flies, either genetically with TRPA1 in dopamine neurons or mechanical disruption, impairs VA1v development.

2.2. Microbiome and sleep

2.2.1. What is the Microbiome?

Though it has been a long accepted fact that all animals are chronically infected with microorganisms, their presence only recently started to gain the deserved attention. The development of faster sequencing methods and drastic reduction in their cost paved the path for an in depth analysis of these microbes within bodies. In the last decade a great number of studies aimed to understand the dynamics of these host- microbe interactions, and how these interactions might influence the health of an animal. Besides common gastrointestinal (GI) disorders, such as Irritable bowel syndrome (IBS) (Kennedy 2014; Collins 2014) and Crohn's disease (Gevers et al. 2014; Hofer 2014), several complex neurological disorders, such as schizophrenia, autism were also shown to come along with GI tract pathologies (Vandvik et al. 2004; Sampson & Mazmanian 2015). Moreover few studies reported that commensal bacteria might also influence behavior, and mood, by altering neurotransmitter release and been therefore linked with conditions such as anxiety and depression (Bravo et al. 2011; Borre et al. 2014; Clarke et al. 2014).

Microbiome composition is highly dynamic and is influenced by a number of factors. A dysbiosis: microbial imbalance may be caused by several factors; such as host genetics, lifestyle and medical practices (Round & Mazmanian 2009). Individuals with mutations in genes involved in immune regulatory mechanisms demonstrate an uncontrolled intestinal inflammation, which consequently influence the bacterial composition (Hampe et al. 2001; Goodrich et al. 2014). However the leading factor that leads to dysbiosis is not genetics, but lifestyle. Diet, stress, the overuse of antibiotics and vaccination are the major factors underlying the rapid increase in dysbiosis related diseases.

The Human Microbiome Project (HMP) began in 2008 as an extension of the Human Genome Project. Main goal of HMP is to map and characterize the combined genomes of all the microbes in the human body. With about 10^{14} microorganisms, human GI tract is the largest reservoir of microbes in the

body. Over 99% of gut bacteria are anaerobic bacteria, with rest 1% being archeabacteria, fungi and protozoa. Bacterial composition is highly variable between individuals and changes in lifestyle, diet and age also alter the microbial dynamics. The human gut harbors approximately 500-1000 bacterial species (Eckburg et al. 2005), with Firmicutes, Bacteriodetes, Actinobacteria and Proteobacteria being the four dominant phyla (Ley et al. 2006; Ley et al. 2008). The two dominant phyla in the gut are Bacteroidetes (17-60%) and Firmicutes (35-80%) (Balzola et al. 2010; Shoaie et al. 2013)

The first exposure to microorganisms occurs during birth from mother's genital tract and at the age of two the microbial profile is mature and stabilizes to a "adult-like" microbiota. (Palmer et al. 2007; Fanaro et al. 2003). The dominance of Bacteriodetes and Firmicutes and a low abundance of Proteobacteria and gram-negative bacteria define the "adult- like" human microbiome. Interestingly old age microbiome showed a greater variability than that of the younger adults (Claesson et al. 2011; Claesson et al. 2012)

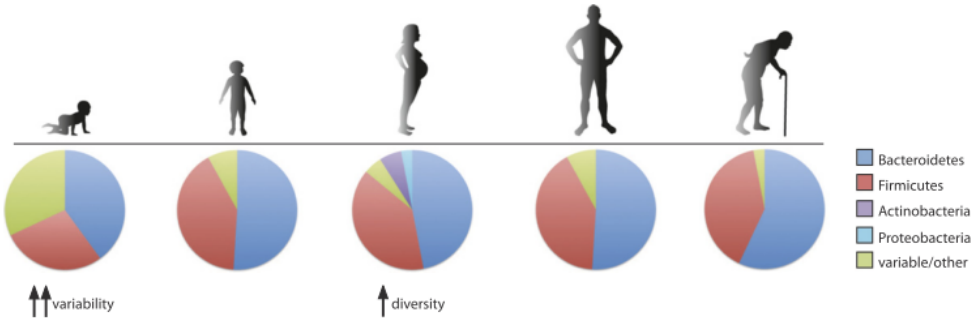

Figure 10. The composition of the human gut microbiota across the course of life (adapted from Kostic et al. 2013)

2.2.2. Microbiome studies in animal models

Current host-microbiome studies mostly employ vertebrate models. Studying germ-free animals showed that the presence of a healthy gut flora is essential for the maturation of the intestinal track. More importantly gut flora plays an important role in the development of gut immunity. Recently

Germ free mouse models have been the main experimental system for microbiome studies. Taxonomically mouse microbiome is highly similar to

humans, and so far the best studied one. Gnotobiotic mice are delivered by sterile Caesarean sections and are kept in sterile isolators, which are constantly ventilated with sterile filtered air under positive pressure (Macpherson & Harris 2004). As expected mice born by caesarean section hosted a different set of bacteria than naturally born mice (Bravo et al. 2011; Heijtz et al. 2011). Studying the behavior of these mice revealed the first indices for the importance of early life microbiome. Mice delivered by C-section displayed depression symptoms and were significantly more anxious than naturally born mice.

However raising mice under these conditions is laborious and costly. Therefore model systems, which are easier to rear under sterile conditions, such as Drosophila (Blum et al. 2013) and Zebrafish (Kanther & Rawls 2010; Rawls et al. 2006) started to gain more interest. Another remarkable model system for microbiome research is the Hawaiian bobtail squid (*Euprymna scolopes*), which is in symbiosis with only one bacterial species, *V. fischeri.* V. fischeri is required for the development of the light organ, as squid raised in a

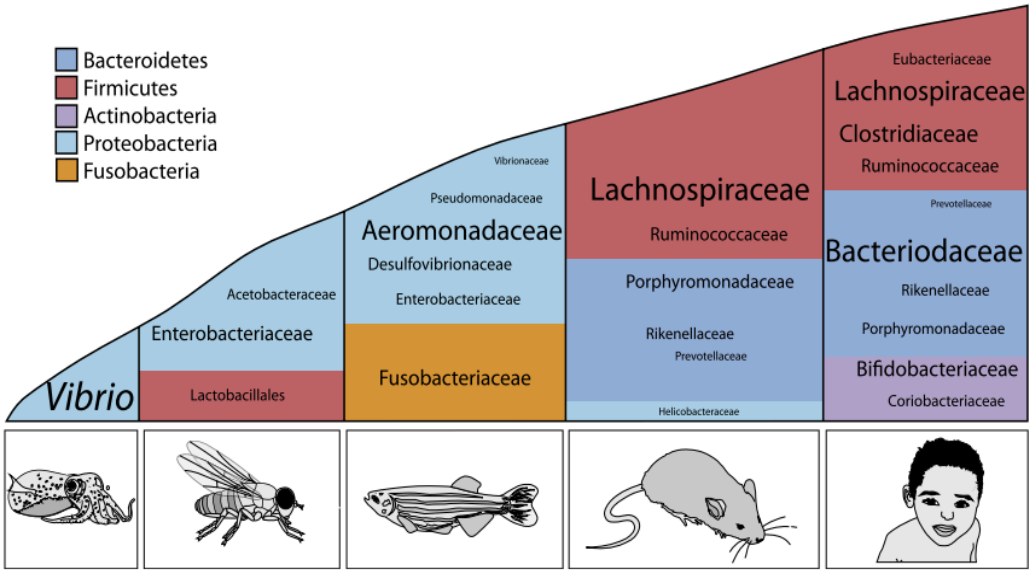

Figure 11. Microbiome composition across species (adapted from Kostic et al. 2013)
Hawaiian bobtail squid has the simplest microbiome composition with only one species, *Vibrio Fischeri. Drosophila* and *Danio Rerio* microbiome are relatively simple with 5-30 species, but shows great variance depending on diet. With mouse and humans the interspecies variation of bacterial composition gets more complex, but there are broad trends at the phylum level.

sterile environment without V. fisheri failed to develop the mature light organ (McFall-Ngai & Ruby 1991; Mcfallngai & Ruby 1998).

Drosophila is emerging as a strong model system, offering several advantages over the other model systems. The most important advantage is the greatly reduced microbial composition. The fly gut hosts five to twenty commensal species, with *Acetobacteraceae* and *Lactobacillaceae* being the prominent families. The five major bacterial species in Drosophila gut flora are; *Commensalibacter intestini*, *Acetobacter pomorum*, *Gluconobacter morbifer, Lactobacillus plantarum*, and *Lactobacillus brevis* (Shin et al. 2011).

Drosophila embryos are sterile, however their eggshells are contaminated with bacteria from feces of adults. Thus *Drosophila* microbiome is acquired after larval hatching, by ingesting the chorion of embryos (Bakula 1969). Similar to the observation in humans, the microbial composition of *Drosophila* is dependent on age(Ren et al. 2007; Kostic et al. 2013). Bacterial density increases throughout larval stages (Bakula 1969; Storelli et al. 2011). During pupation the bacterial density fluctuates (Tryselius et al. 1992). This fluctuation is associated with the increased expression of antimicrobial peptide genes (Tzou et al. 2000). Newly hatched adult flies have quite low bacterial counts, ranging from 40- 1.000 cells per gut. Aging leads to a significant increase in both external and internal bacterial density, up to 10-1000 fold (Ren et al. 2007). Moreover as observed in humans, there is a shift in bacterial composition as well (Wong et al. 2011).

As in humans and mammals, *Drosophila* diet is also the major factor that influences the bacterial composition of gut microbiome. Consequently there is a great variance in the bacterial composition of wild-caught flies but also in flies between different laboratories.

Besides age and diet, genetic factors also influence the fly microbiome. So far, only few studies analyzed the impact of host factors on the gut microbiome composition. One study showed that flies with higher levels of antimicrobial peptides (AMPs) in the posterior midgut, presented an increase of a minor member, *Gluconobacter morbifer* and a decrease in a dominant member *Commensalibacter intestine* (Ryu et al. 2008). Another study reported that Relish and PGRP-LC flies, which had impaired Imd pathway

activities had higher bacterial counts in their gut (Buchon et al. 2009; Broderick et al. 2014)

2.2.3. Microbiota- gut- brain axis in mammals and Drosophila

Gut bacteria can interact with the nervous system through several pathways. A direct activation is achieved via vagus nerve from the ENS to CNS. A more complex and indirect interaction occurs through circulatory system. Intestinal bacteria have been shown to be producing various metabolites and also metabolic precursors to hormones and neurotransmitters. Another interaction is via Immune system, where bacterial metabolites can signal and alter the immune response. (Round & Mazmanian 2009).

But how can these pathways interact with sleep regulatory systems? The link between gut microbiota has not been studied yet. However, all three pathways of gut-brain axis are also known to be involved in sleep regulation (Marshall & Born 2002; Armitage et al. 2003; Galland 2014)

One particularly attractive hypothesis for microbiome and sleep regulation is via immune system. The intestinal microbiota is shown to be actively

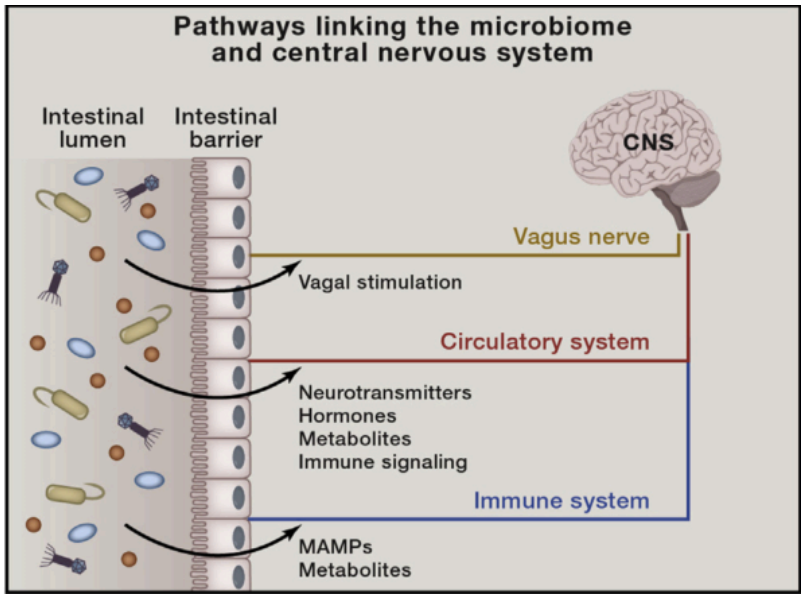

Figure 12. Brain-gut axis: Pathways linking the microbiome and central nervous system (adapted from Sampson & Mazmanian 2015)
(1) Direct pathway via vagus nerve
(2) Indirect activation via circulatory system, achieved through metabolites and metabolic precursors produce by intestinal bacteria.
(3) Immune system activation through metabolites or MAMPs such as LPS, BLP and PSA

regulating host immunity and the intestinal homeostasis is dependent on the immune cells induced by a healthy microbiome. The bidirectional interaction between immune system and the CNS in mammalian systems is well established (reviewed in Dantzer et al. 2008). Moreover sleep loss is known to alter immune function and vice versa immune system disturbances can interrupt healthy sleep. Immune signaling molecules, called cytokines have been extensively studied for their involvement in sleep regulation.

The link between drosophila brain and gut microbiome is currently not very well understood. There is probably no direct activation, however it is intriguing to hypothesize that the indirect pathways either via metabolites produced by intestinal bacteria or through immune system activation are present in flies as well.

Drosophila has two gut immune effectors, which are known to control gut microbiota: DUOX and IMD-Relish. Duox activation induces production of ROS. DUOX over activation due to high level of bacterially derived uracil leads to an excess ROS production, which causes oxidative damage to host.

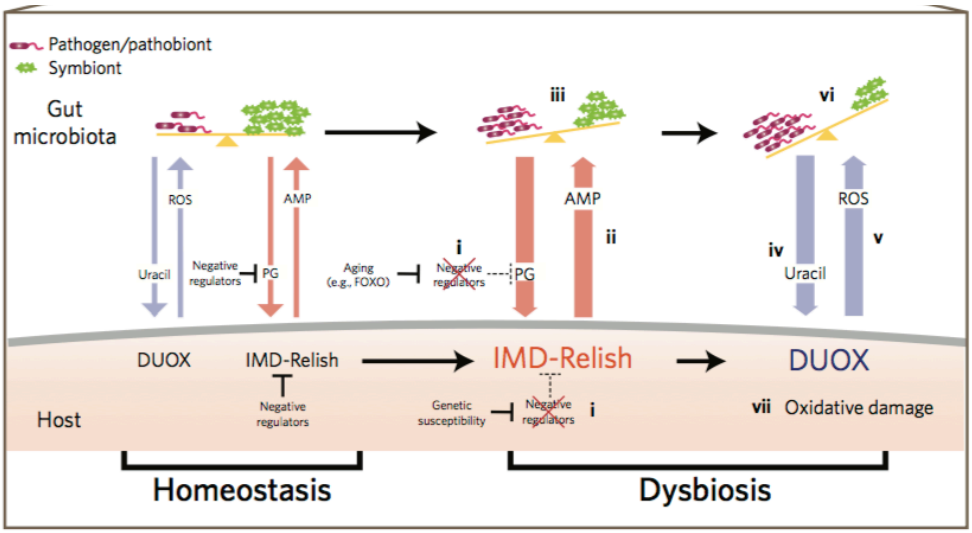

Figure 13. A model for homeostasis and dysbiosis of *Drosophila* gut adapted from (Lee & Hase 2014)
IMD-Relish and Duox are activated upon bacterially derived metabolites. These produce two different effectors: AMP and ROS, which establish the bacterial homeostasis. A dysbiosis can occur upon loss of negative regulation, due to genetic factors or as a consequence of aging-dependent changes.

Similarly the loss of negative regulators of Imd-relish pathway leads to an enhanced AMP production. Dysbiosis might also occur upon aging-dependent changes in immune regulation, e.g. altered FOXO activation) or due to mutations impairing negative regulation.

What are the consequences of dysbiosis and can they affect sleep regulatory mechanisms? These questions have not been addressed yet but considering the literature one intriguing hypothesis is an AMP dependent pathway. A recent study identified changes in levels of anti-microbial peptides (AMPs) in sleep-depreived flies. *Metchnikowin (Mtk)* and *drosocin (dro)* mRNA transcript levels were significantly increased in sleep deprived animals. *Dro* showed an increase in neurons, whereas Mtk only in glia. These results were further confirmed with rescue experiments, by using either a glia specific or a neuron specific driver.

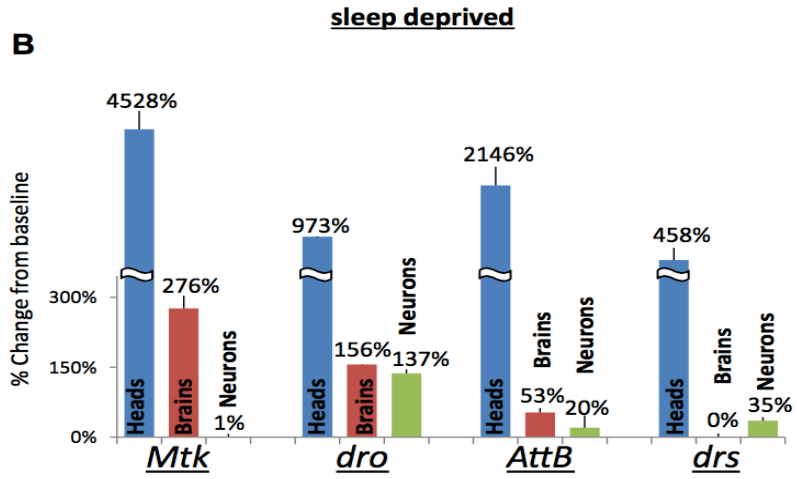

Figure 14. Sleep deprivation increases levels of AMPs (Dissel, Seugnet, et al. 2015)
The mRNA extracted from whole heads, brains or neurons were analyzed. All 4 AMPs showed a significant increase in whole head mRNA transcripts but only Mtk and dro were specific for neurons and glia.

2.2.4. Alzheimer's disease microbiome and sleep

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common cause of dementia in the world (Wisniewski & Goñi 2014). Only a small fraction of AD has familial inheritance, with rest having sporadic etiology (Tanzi 2013). Familial forms of AD are associated with mutations in amyloid precursor protein (*APP*) and presenilin 1 and 2 (Karch et al. 2014). The major influence causing AD pathology is considered to be the accumulation of β-Amyloid (Aβ) peptides within the brain (Hardy & Selkoe 2002). Interestingly cognitive impairments associated with AD are frequently accompanied by sleep problems (Mander et al. 2015; Peter-Derex et al. 2014). Patients with AD have reduced and fragmented sleep at night and there are few studies suggesting that poor sleep might be a contributing factor for the pathology (Ju et al. 2014; Peter-Derex et al. 2014).

Drosophila models of AD are commonly used to understand the genetics of disease dynamics. There are various transgenic constructs, which model different aspects of AD pathogenesis; e.g. Aβ accumulation, tau toxicity (Fernandez-Funez et al. 2015). Moreover the *Drosophila* APP orthologue *Appl* shares the basic structure of human *APP* and Appl deficient flies are partially rescued by the expression of human APP (Luo et al. 1992; Poeck et al. 2012). Conveniently AD pathology in fly models is associated with various phenotypes, which are relatively easy to assess; e.g. shortened lifespan, cognitive function deficits, and abnormal locomotor behaviour. Interestingly these phenotypes are reminiscent of symptoms observed in human patients.

Two recent studies highlighted the importance of utilizing *Drosophila* to investigate the link between sleep and AD(Dissel et al. 2015; Tabuchi et al. 2015). First one demonstrated the link between sleep loss and Aβ burden and found that the sleep promoting properties of the fan shaped body were disrupted in AD models, due to neuronal hyperexcitation. Additionally they showed that reducing the neural activity in AD flies by using a anticonvulsant levetiracetam (LEV) prolonged their lifespan.

Another interesting study showed that inducing sleep via THIP, a sleep promoting GABA-A agonist, could reverse memory deficits in three different models of AD (Dissel et al. 2015). Thus, taken together both these studies

emphasize the role of sleep in AD and suggest the idea that increasing sleep may slow down the progression of AD or even better it may improve the cognitive deficits.

Recently the contribution of microbiome to human neurodegenerative diseases is gaining more recognition (Galland 2014). Interestingly microbiome bacteria are also shown to produce amyloid peptides and lipopolysaccharides (LPS), which may contribute to amyloid burden (Zhao & Lukiw 2015; J. M. Hill et al. 2014). Additionally there is a growing list of bacteria, which are being implicated in AD pathogenesis (Hammond et al. 2010; Balin & Hudson 2014) and remarkably there are numerous similarities between changes seen in microbial infections and AD (Cho & Blaser 2012).

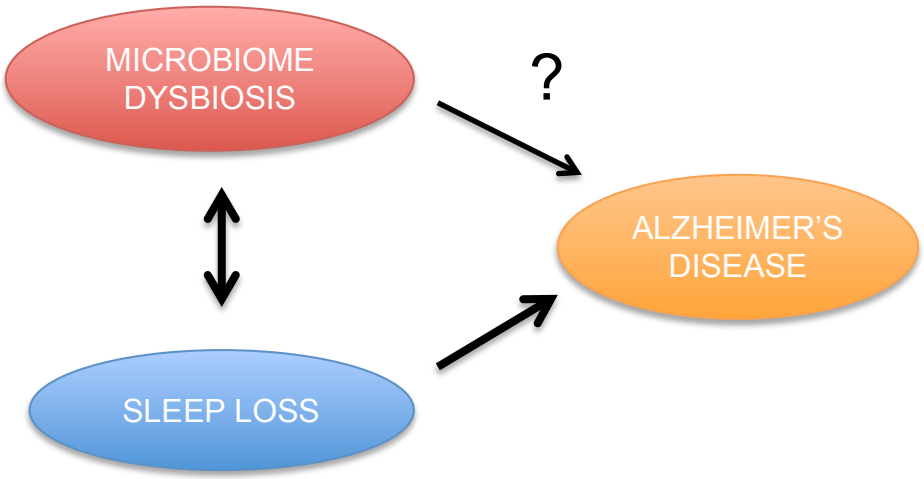

Figure 15. Model for the possible microbiome-sleep related AD pathology.
The influence of microbiome on AD pathology is gaining more support due to several observations (Bhattacharjee & Lukiw 2013). The most important one is that microbes of the human microbiome secrete amyloid, which is the primary influence driving AD. Sleep disturbances in AD patients are common, how or whether sleep loss is a contributing factor for AD pathology is currently unknown. However there are several indices, which suggest that sleep can be applied as a therapeutically approach (Ju et al. 2014; Peter-Derex et al. 2014; Dissel, Angadi, et al. 2015). Understanding the dynamics between microbiome and sleep, will also give us better clues on how these lead to AD pathology.

2.3. Aim of this study

Microbiome research is at its booming era and there is a growing literature on how the bacteria in our gut might act like a “second brain”. This study aimed to investigate the significance of early age microbiome on adult life sleep in *Drosophila*.

Young age is a fragile stage, and is especially important for the developing brain. The increase in early life sleep among most animals suggests an important role for sleep. Sleep in young flies presents the same phenotype as young Drosophila sleep significantly longer than adult flies. Moreover sleep disturbances during this period resulted in long lasting learning deficits (Seugnet et al. 2011) and deficiencies in adult courtship behavior (Kayser et al. 2014). Similar importance is considered to be true for early life microbiome. First indices to support this idea came from mice studies, where where mice born by caesarean section hosted a different set of bacteria than naturally born mice. These mice displayed depression symptoms and were significantly more anxious, than naturally born mice(Bravo et al. 2011; Heijtz et al. 2011)

Drosophila has been proven to be a great model system for studying sleep. Although microbiome field is relatively new for *Drosophila*, the advantages of genetic techniques and behavioral assays offer a great system to investigate the brain-gut microbiome link.

3. Materials and Methods

3.1. Materials

. Sodium hypochlorite solution 10-15% (Sigma Aldrich, CAS-Number: 7681-52-9) http://www.sigmaaldrich.com/catalog/product/sial/425044?lang=en&region=US

- Ethanol (Sigma Aldrich)
- Propionic acid (Sigma Aldrich)
- Tetracycline (Sigma Aldrich)
- Penicillin (Sigma Aldrich)
- Streptomycin (Sigma Aldrich)
- Mold inhibitior (Methyl paraben, Tegosept) (Sigma Aldrich)

3.2 Methods

3.2.1 Fly Strains

Canton-S

w^{+};;$Relish^{E20}$, e^{+}/ TM3;

yw ; ; Rel^{E23};

Rel^{E26}.TM3;

Rel^{R6e}/ TM3

IMD^{1}

Gr23 Gal4

UAS APP BACE;

3.2.2 Maintenance and crossing of fly stocks

Fly stocks were kept on Drosophila standard food in a 25^{o}C room or in incubators with 12:12 light dark cycle and 60% humidity.

For crosses newly hatched virgin females were crossed with males of various ages. All crosses were performed at 25^{o}C.

3.2.3 Fly pushing

Flies were anesthetized with carbon dioxide on C02 pads. However in order to avoid the detrimental effect of anesthesia, flies were transferred to behavior chambers with the help of an aspirator.

3.2.4 Binary expression systems (Gal4/Uas, LexA/LexAop)

In a binary expression system a fly stock expressing a trans-activator or driver is crossed with a fly bearing a responder element, responder line. As a result the progeny (F1) of such a cross expresses the gene of interest at desired cells where the driver is expressed.

The Gal4/UAS system, with yeast transcription factor Gal4 and its upstream activator sequence (UAS), was the first binary system to be used in Drosophila (Brand & Perrimon 1993).

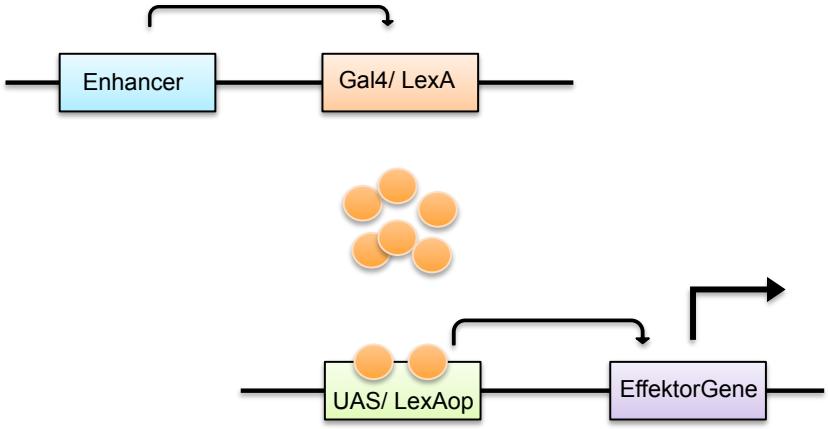

Figure 16. Principle of bipartite systems: Gal4/UAS and LexA/LexAop
This system relies on two components; (1) a driver line under the transcription factor Gal4 or LexA, (2) an effector line with a transgene under the control of UAS or LexAop. The two components of this system are combined via a genetic genetic cross with these lines. In F1 of this cross the gene of interest is only expressed in the cells expressing the Gal4 or LexA protein.

3.2.5 Fly food recipe

Fly food was prepared every two weeks. Day before the cooking procedure yeast was autoclaved for 45 minutes on wet cycle (Cycle number: liquid 6)

Final amount in litres:	Calculated for 30L
Yeast (100% Sci Mart) in grams	1500
Tap water for autoclaving yeast:	10
Sucrose (g)	450
Karo corn syrup (mL)	900
Molasses (mL)	999
Agar (g)	270
Tap water added:	20
Proprionic Acid	112,5
Mold inhibitor (15g/100mL)	225
Antibiotics (5%) (DOXY/GENT or Pen/strep) (mL)	30

- “Regular Antibiotics” food is made with Pen/Strep at a concentration of 5%- 1ml antibiotic per liter of food.

- “High Antibiotics” food is made with pen/strep, gentamicin, doxycycline, tetracycline and ampicillin at a concentration of 5% per liter of food (except tetracycline, which is 1mL per 5L of food).

3.2.6 Sterile food and egg collection plate preparation

- Sterile food preparation

An aliquot was taken from the cooked food; before mold inhibitor, propionic acid and antibiotics were added. 10%v/v water was added before the autoclaving procedure. Food, tubes and plugs were autoclaved for 30 min. at wet cycle (cycle number: liquid 5). Autoclaved food was cooled down to a temperature of approximately 30-40 Celsius. Mold inhibitor, propionic acid and antibiotics were added in the same concentration as in the original recipe.

- Preparation of apple juice agar plates

1% agar was added to water apple juice mix (50:50v/v). The mix was put into microwave and boiled until agar was completely dissolved. Mix was cooled down and poured into petri plates.

- Preparation of yeast paste plates

Tap water was added to inactivated yeast and the mix was stirred until desired consistency. Petri plates were filled with the paste right before egg collection.

3.2.7 Wolbachia Elimination protocol

In order to eliminate the natural endosymbiont, *Wolbachia pipientis*, flies were placed on a high tetracycline food for three generations. After three generations on high tetracycline food (0.25 mg/ml tetracycline in 75%Ethanol), flies were then transferred to vials with normal food, with regular antibiotics.

3.2.8 Axenizing protocol

Freshly laid eggs< 18 hours were collected from the plates with the help of a brush. They were then transferred to cell strainers and the eggs were washed briefly with sterile water to remove the sticky agar.

Both axenization and mock treatments were performed under the hood. Plastic cuvettes were filled with freshly prepared 2.5% bleach, 75% ethanol and sterile water. Both treatments were performed simultaneously for the same genotype.

For axenization treatment eggs were washed in 2.5% bleach for 5 minutes, then transferred to 75% Ethanol for a brief wash and then washed thoroughly with sterile water. Mock treatment was done with sterile water. (Figure x) (Protocol adapted from Storelli 2015)

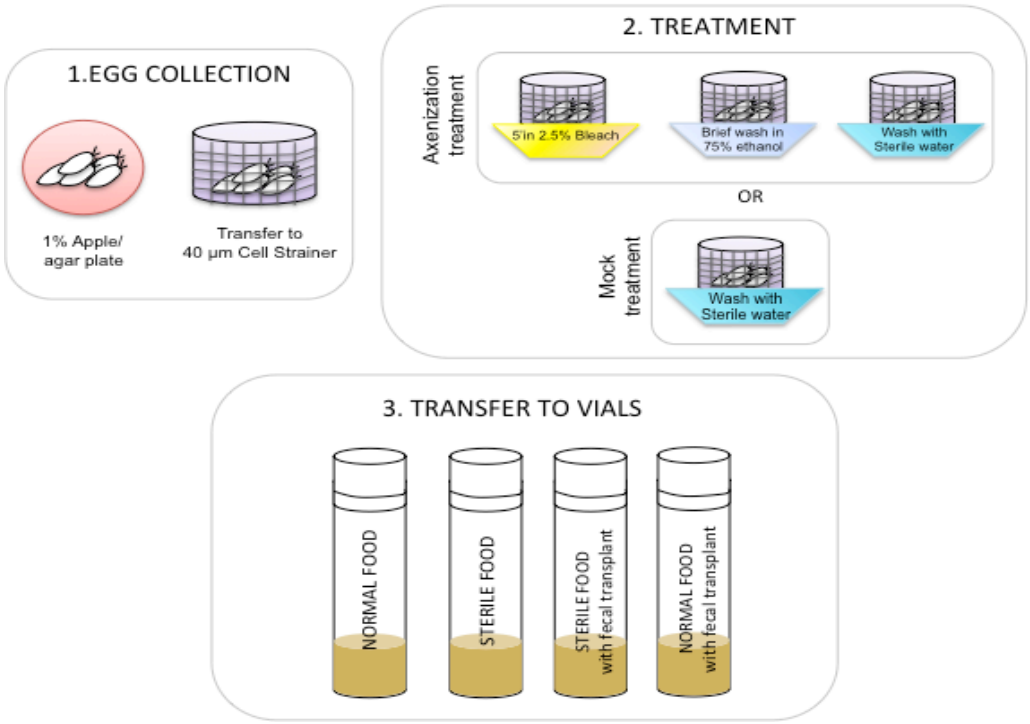

Figure 12. Protocol for axenization and mock treatment

1. Egg collection was performed on 1% apple/agar plates or on yeast paste plates. Eggs were gently loosened from the agar with a paint brush. Loosened eggs were transferred to a cell strainer and briefly washed with sterile water to remove the agar sticking on the eggs.

2. eggs were seperated into two groups and one group received the dechorionation (Axenization) treatment, wherease the second group received a mock treatment (sterile water washes).

Both treatments were performed simultaneosly and under the laminar flow hood.

3.Eggs were then transferred to fresh vials with regular food or sterilised food.

3.4 Behavioral Analysis

3.4.1 Sleep recording (Dam system Trikinetics)

Two to three-day-old flies were placed into 65mm transparent glass tubes with a piece of food. The activity of flies was tracked by using the Trikinetics (www.trikinetics.com) activity monitoring system as previously described (Shaw et al. 2000).

5- x 65-mm glass tubes are filed with food on one side and sealed with wax. The opposite end is sealed with a piece of cotton. Tubes with individual adult flies are loaded into activity monitors. An infrared emitter/detector records the activity of the fly, more specifically it counts each time a fly crosses the center of the tube. Connected computer interface continuously records the data.

Locomotor activity was measured in 1-minute bins and sleep was defined as periods of quiescence lasting at least 5 minutes.

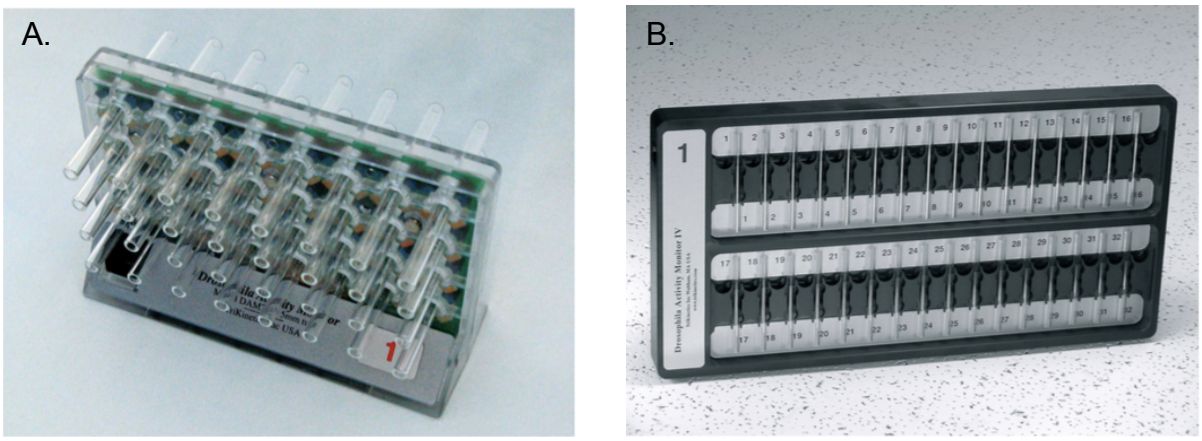

Figure 13. Drosophila activity monitors (images from www.trikinetics.com)

A. DAM2 System: 32 tubes, with dual IR beams per tube, and integrated on/off ambient light sensor.
B. Dam5 System: 32 tubes, with single IR beam per tube

Both systems were used for data acquisition

- Protocol for analyzing sleep data

Sleep data was analyzed with Microsoft Excel. Following data was extracted from the program for further analysis.

- Latency
- Total sleep time in minutes
- Day bout
- Max day bout w
- Night bout
- Day bout number
- Night bout number
- Max sleep time
- Count per minute

3.4.2 Sleep nullifying apparatus

The Sleep Nullifying Apparatus (SNAP) was used for sleep deprivation experiments. The apparatus tilts asymmetrically from -60° to +60° and thus sleeping flies are displaced during the downward movement 6 times per minute (Shaw et al. 2000; Seugnet et al. 2008).

3.4.3 Courtship Behavior

Drosophila courtship behavior is a very robust innate behavior. Drosophila courtship conditioning is a form of associative learning, and behavior of males is modified by previous sexual experience. Males, who are exposed to a previously mated female get rejected and therefor they suppress their courtship behavior for up to 3 hours. The strength of suppression is measured by the courtship index, which is calculated by the total amount of time a male fly spends courting divided by the total duration of a testing period.
Drosophila courtship conditioning is a powerful tool to study learning and memory. Yet analyzing the assay is time consuming and tedious, therefor it is necessary to automate process.
There are few open software programs available, yet these are still to be improved. A new software, called as Actual track came on market in 2014, which was developed to analyze a wide range of behaviors of different animals.

- Optimization of video acquirement for Actual track analysis
One problem we encountered was the uneven light conditions during video acquisition. We minimized this problem by using a soft white light (Huion L4S Tracing LED Light Pad, 1100 Lux), which illuminated the arena from below. This illumination allowed a great amount of contrast between background and subjects and improved the video quality greatly (Figure B) .

- Video processing for Actual track analysis
The tracking software depends mainly on the contrast between background and subjects. In order to optimize the recorded videos I tested several video effects and color correction settings templates by using Movie maker (Microsoft Windows) (Figure C). Highest tracking accuracy was achieved when inverse color template was applied.

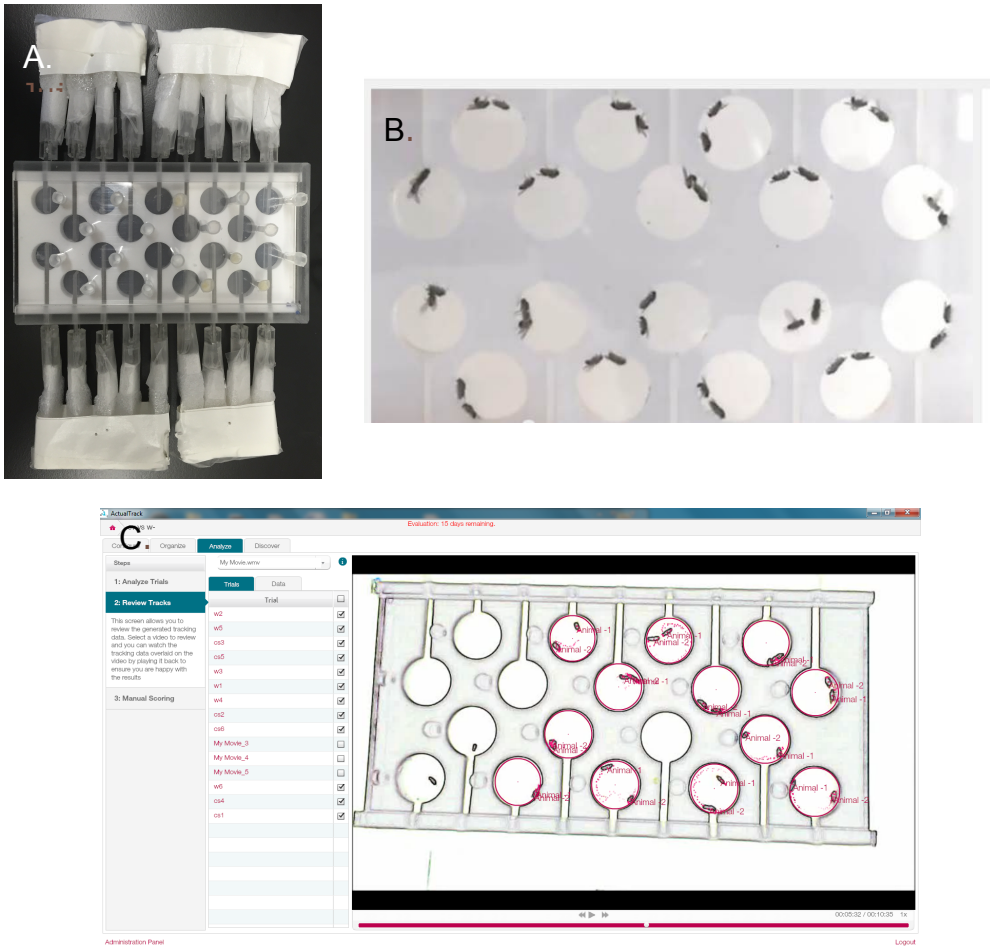

Figure 20. Courtship assay chamber and analysis software

A. 18-Well courtship arena, each chamber is divided by a separator.
B. Snapshot of an ongoing test
C. Snapshot of the analysis software

3.5 Statistics

All comparisons were done using a Student's T-test. All statistically different groups are defined as $p < 0.05$.

Symbol	P-value
n.s.	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$

4. Results

4.1 Wolbachia and Sleep

Wolbachia Pipientis infection has been found in a great number of arthropods (Hilgenboecker et al. 2008). *Wolbachia* is a gram-negative, intracellular bacterium, which is transmitted via maternal germ line (Serbus et al. 2008; Werren et al. 2008). The dynamics of this symbiotic relationship and its benefits for the host is of great interest. One interesting observation was that some Drosophila species, such as *D. Simulans* showed strong cytoplasmic incompatibility (CI) when infected with *Wolbachia* (Bourtzis et al. 1996). Whereas in *D.Melanagoster* CI is almost completely repressed. *D.Melanogaster* brain has been shown to be infected with *Wolbachia* as early as in larval stages (Albertson et al. 2009). The densest infection is found in the central brain, a brain region, which plays a critical role in regulation of various behaviors. Therefore it is intriguing to hypothesize that *Wolbachia* infection might result in behavioral outcomes (reviewed in Albertson et al. 2013). One example for a behavioral consequence of *Wolbachia* infection was described in Rohrscheib et al. 2015, where a specific strain of *Wolbachia* infection reduced aggression in male fruit flies. The question whether this symbiotic relationship is required to maintain a healthy sleep was however not addressed. (Published during my research stay by Vale & Jardine 2015).

Therefore I compared the sleeping patterns of regular CS flies and *Wolbachia* free CS flies (W-). *Wolbachia* elimination treatment is described in materials and methods section. Briefly, flies were treated with tetracycline for three generations. I observed normal sleeping patterns in both groups for both males and females. CS (W-) female flies had a slight increase in night bout. However this is likely a response to the dietary change, as CS (W-) flies were transferred to regular food post tetracycline treatment for 2 days.

These results are in partial agreement with the recently published paper (Vale & Jardine 2015). This paper suggests a protective effect of *Wolbachia,* which is more evident in males, as W- male flies showed reduced activity compared to W+males.

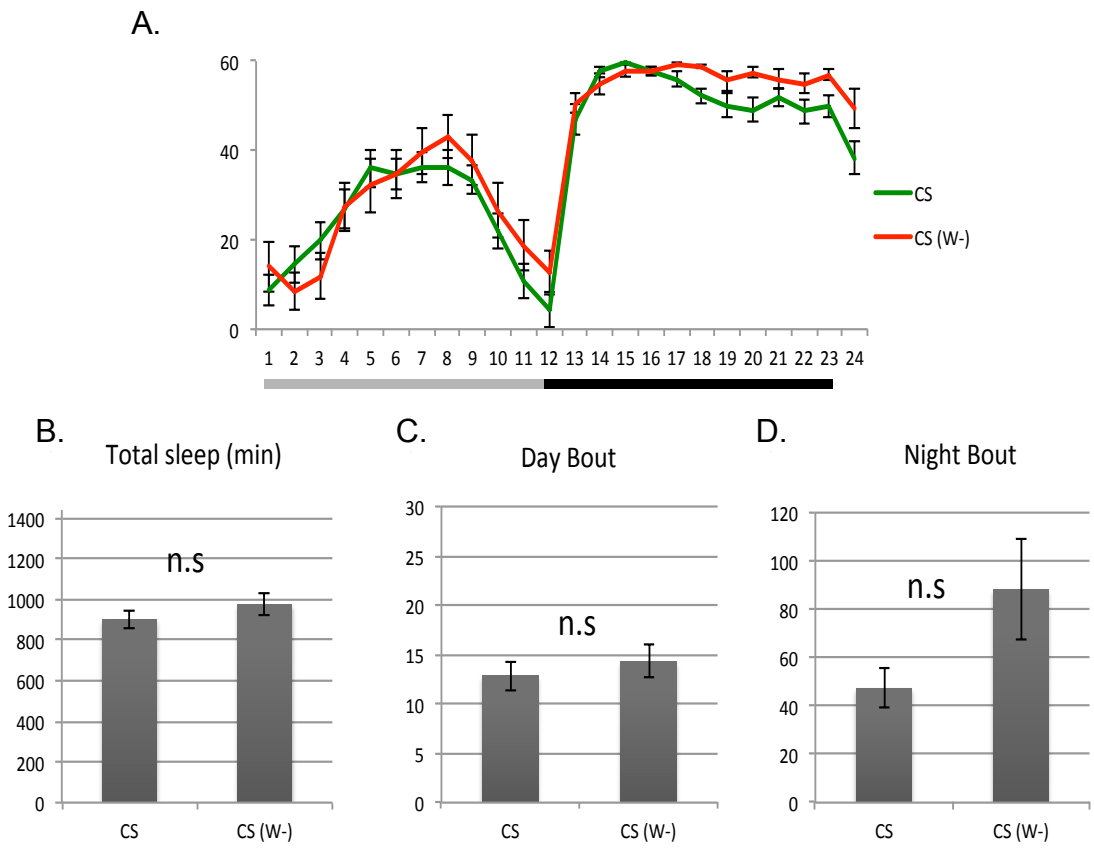

Figure 21. Sleep analysis of *Wolbachia* free (W-) Canton-S flies

Sleep time was not significantly different between flies, which were *Wolbachia* free, and their siblings, which were untreated.

(A) Daily sleep in min/hour at age 4-days in CS and CS(W-). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between two groups. (C) Average sleep bout duration during lights on (day bout) was not significantly different between two groups. (D) Average sleep bout duration during lights off (night bout) was not significantly different between two groups.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

4.2. Sleep profile of axenic wild-type CS flies before tetracycline treatment

I analyzed the sleep behavior of CS (W+) flies, which were untreated for *Wolbachia*. Three groups of flies were analyzed; untreated flies, mock treated flies and dechorionated flies (germ-free). The axenization protocol is described in materials and methods section. Briefly, axenized eggs were aseptically transferred to vials, either with regular food or sterilized regular food. After hatching, female flies, if possible virgin females were collected under CO_2 anesthesia and were transferred to a fresh vial, with regular food. Following two-three days of recovery from anesthesia, flies were transferred to clean trikinetics tubes: with either regular or sterile food.

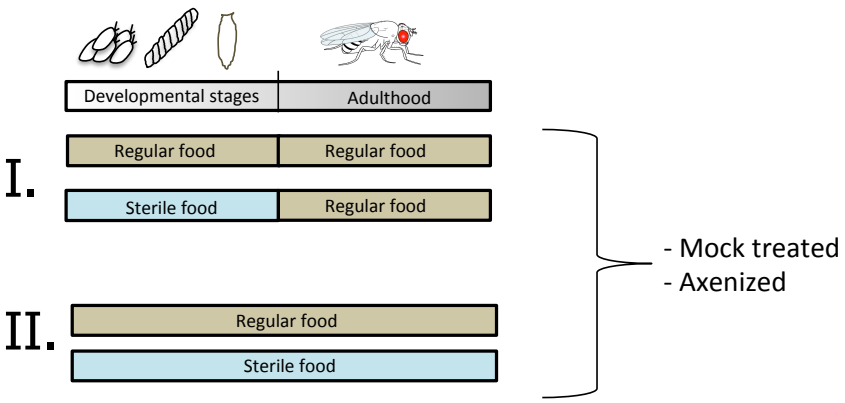

Figure 22. Treatment protocol

I. Flies were transferred to normal or sterile food post mock or axenization treatment. After hatching these were then transferred to fresh regular food vials. After 2 days they were transferred to trikinetics tubes with the same regular food.

II. Flies were transferred to normal or sterile food post mock or axenization treatment. After hatching these were then transferred to fresh regular food or sterile food vials. After 2 days they were transferred to trikinetics tubes with regular food or sterile food. This protocol made sure that the changes we observe are not due to dietary changes.

There were noticeable changes in the food texture after sterilization protocol (sterile food, dried out sooner than the regular food). Interestingly diet dependent alterations in sleep behavior are not very well understood, however have been implicated in few studies (Catterson et al. 2010) Therefore, for all my analyses, I chose to compare the normal food raised and sterile food raised flies separately.

At four days of age axenic flies kept on regular non-sterile food showed similar sleeping behavior as their non-dechorionated siblings Interestingly, they displayed a decrease in night-bout compared to the control group, which persisted at age 5 as well (data not shown).

The axenic flies, raised on sterile food showed a significant decrease in total sleep time and in day-bout compared to their mock treated siblings. Moreover there was a significant decrease in night-bout between untreated and treated (both mock and dechorionated groups).

These data suggest that germ free W(+) CS flies, raised under germ-free conditions have disturbed sleep as adults. Germ free W(+) CS flies raised on non-sterile regular food, can compensate for the early age microbiome loss. We didn't analyze whether their microbiome profile was similar to their untreated siblings.

Another possibility is the involvement of *Wolbachia*, which might be compensating for the microbiome loss in germ free W(+) CS flies, raised under regular conditions, but not under sterile conditions.

Additionally the phenotype, which we are observing in germ free W(+) CS flies, raised under germ-free conditions might be an artifact resulting from food shift. Although this is highly unlikely, because they were transferred to regular food immediately after hatching, and their sleep was recorded on the same regular food.

To sum up, we observed a significant change in sleep behavior of germ free W(+) CS flies, raised under sterile conditions until adulthood, but not in their siblings, which received the same treatment but were raised under regular conditions.

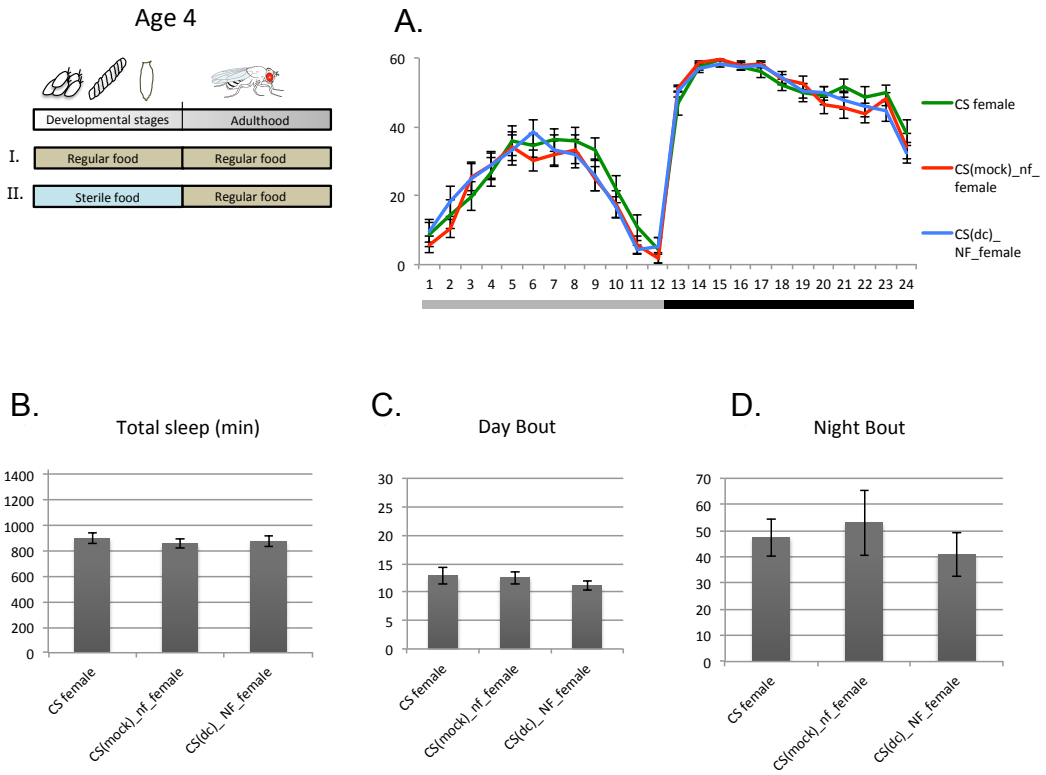

Figure 23. Sleep profile of Canton-S flies (W+) post mock and axenization treatment on normal food (following treatment flies were raised and tested on normal food)

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in CS-untreated, CS-mock treated and CS-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was not significantly different between mock and dc flies. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

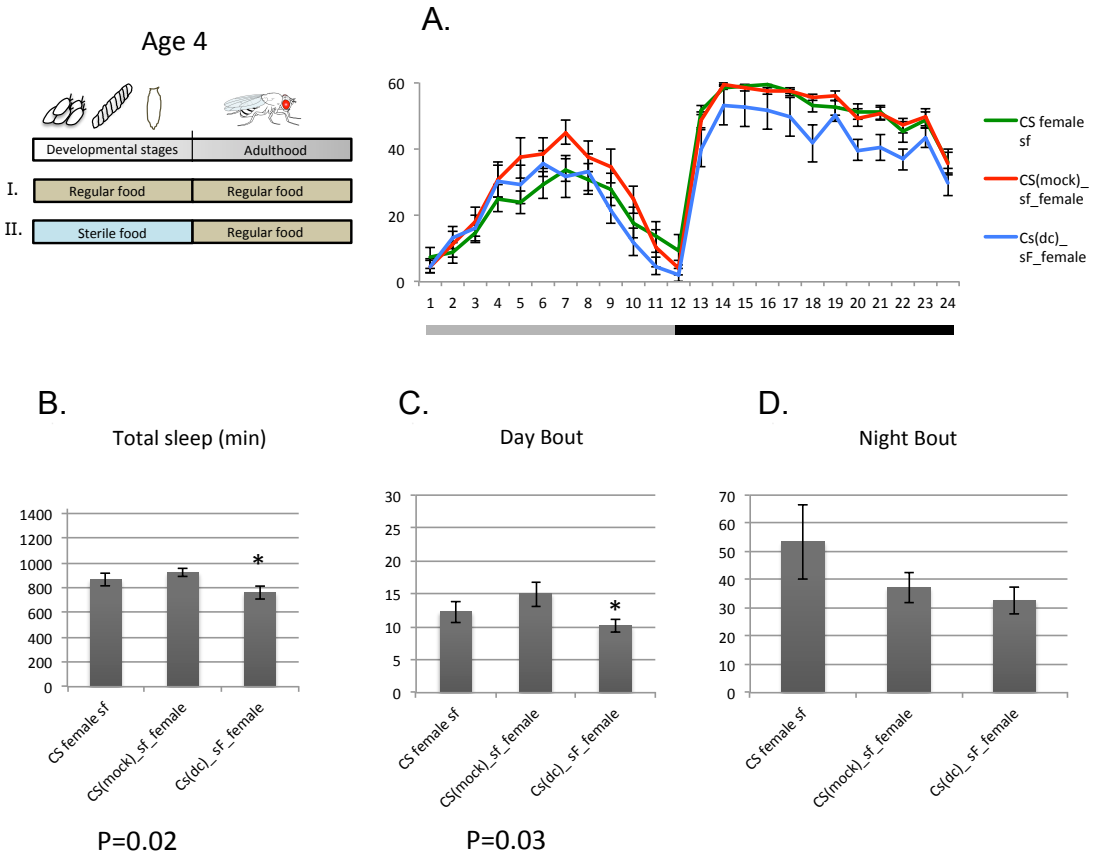

Figure 24. Sleep profile of Canton-S flies (W+) post mock and axenization treatment- on sterile food (following treatment flies were raised on sterile food and tested on normal food)

Sleep time was significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in CS-untreated, CS-mock treated and CS-dechorionated (dc), all raised on sterile food. Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was significantly different between mock and dc flies p=0.02 student's t-test). (C) Average sleep bout duration during lights on (day bout) was significantly different between mock and dc flies (p=0.05 student's t-test). (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies. However both treated groups had significantly lower night bouts compared to their untreated siblings

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

4.3. Sleep profile of axenic wolbachia-free wild-type CS flies (post tetracycline treatment)

I analyzed the sleep behavior of Wolbachia free (W-) Canton-S flies, which were treated with tetracycline, for three generations (detailed protocol in Materials and methods section).

At four days of age axenic (W-) flies kept on regular non-sterile food showed similar sleeping behavior as their non-dechorionated siblings Interestingly, they displayed a decrease in both in day and night-bout compared to the control mock treated group, which persisted at age 5 as well (data not shown). However these changes were not significantly different ($p=0.07$ student's t-test). Interestingly the total sleep time was significantly increased in treated flies, compared with their non-treated siblings. This suggests that applied treatment protocol, although performed in the same way might have an effect on adult behavior.

The axenic flies, raised on sterile food had similar total sleep time compared to both their mock treated and untreated siblings. Interestingly there was a slight increase in daytime sleep between treated group and untreated group, however day-bout between treated groups was similar. Moreover there was a decrease in night-bout in mock treated group, compared to untreated and axenic siblings.

These data suggest that germ free W (-) CS flies, raised under germ-free conditions have no drastic changes in their sleep as adults. Germ free W (-) CS flies raised on non-sterile regular food, as well as germ free W(-) CS flies raised on sterile regular food can compensate for the early age microbiome loss. We didn't analyze whether their microbiome profile was similar to their untreated siblings.

Compared to previous results this further highlights the involvement of *Wolbachia*, which might lead to sleep disturbances in germ-free conditions. To sum up W- flies display no obvious sleeping deficits upon axenization.

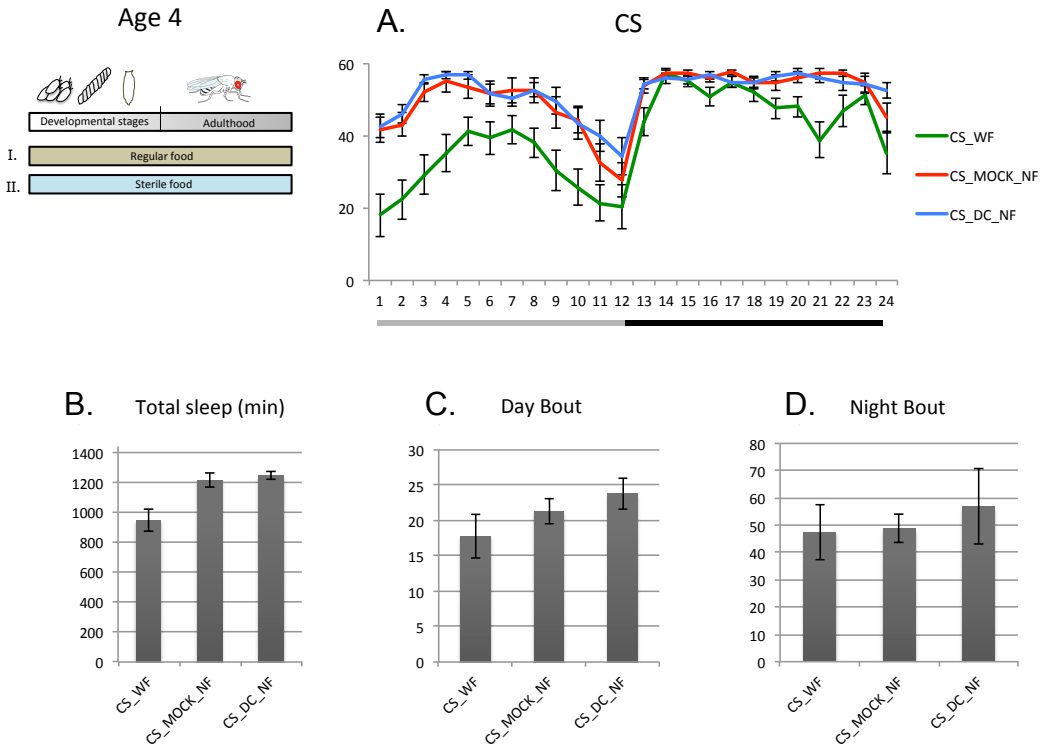

Figure 25. Sleep profile of Canton-S flies (W-) post mock and axenization treatment- following treatment flies were raised on normal food and tested on normal food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in CS-untreated, CS-mock treated and CS-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was not significantly different between mock and dc flies. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

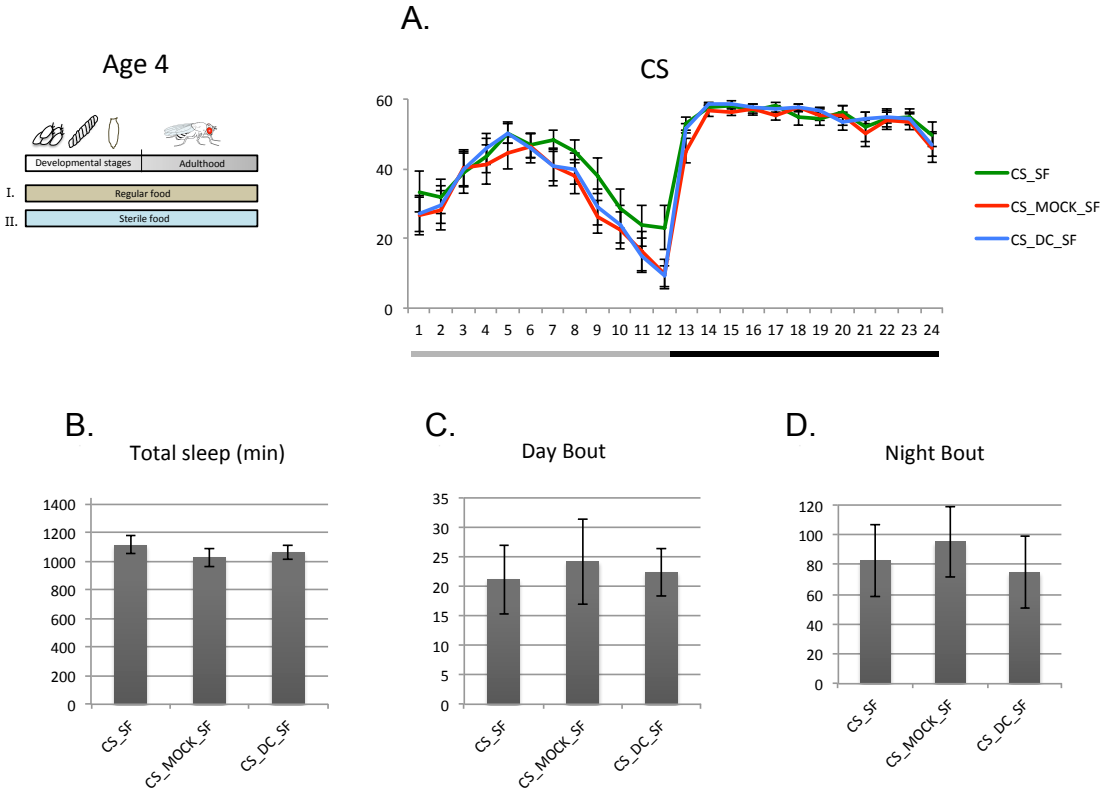

Figure 26. Sleep profile of Canton-S flies (W+) post mock and axenization treatment- following treatment flies were raised on sterile food and tested on normal food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in CS-untreated, CS-mock treated and CS-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was not significantly different between mock and dc flies. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

4.4. Sleep profile of axenic wild-type CS flies upon sleep deprivation

As we observed no obvious differences in the sleep behavior of wild type germ-free and control flies, we asked the question whether these changes could make flies more vulnerable to stress conditions. After recording the baseline sleep for two days, we used a two-day sleep deprivation protocol. Since fly sleep is under homeostatic regulation, sleep deprived flies show an increase in sleep post deprivation (Shaw et al. 2000). We analyzed the sleep recovery on day 2 post sleep deprivation. Flies were 9 days of age on the day of analysis.

Contrary to our expectations germ-free flies displayed no obvious sleep deficits upon sleep deprivation compared to mock treated siblings. Under both conditions, sterile and non-sterile there was a minor decrease in total sleep time and in night bout between untreated and treated flies. However these were not significantly different among treated siblings.

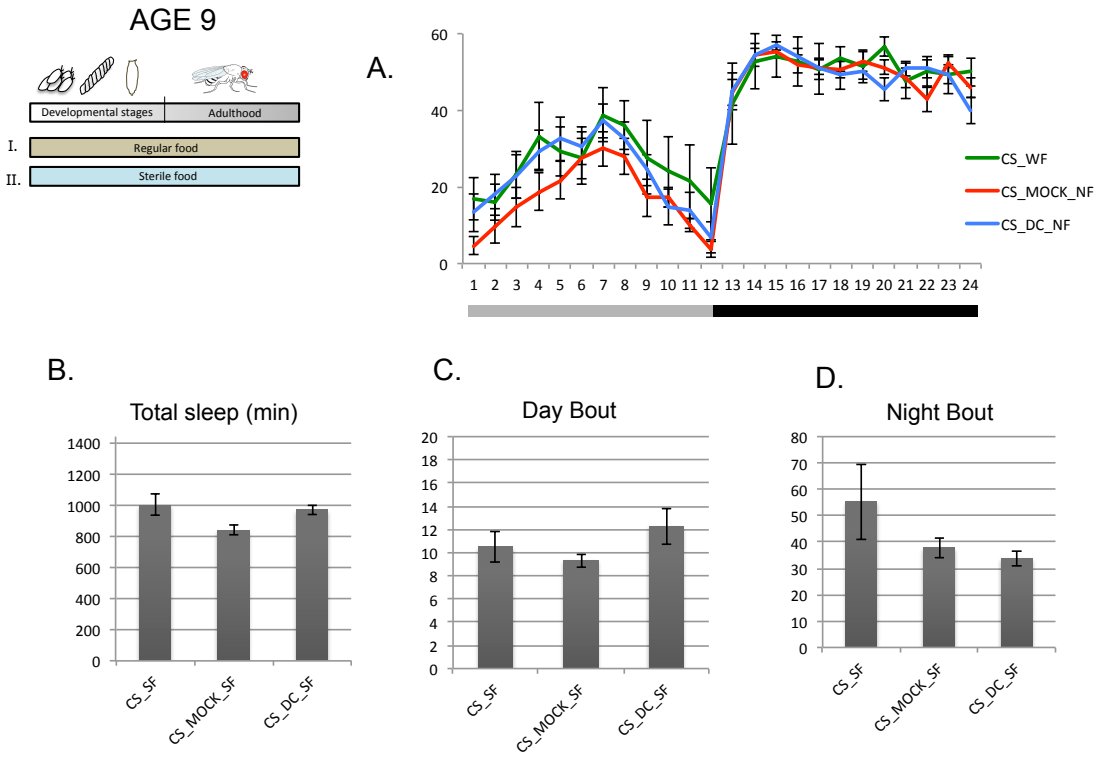

Figure 27. Sleep profile of sleep-deprived Canton-S flies (W+) post mock and axenization treatment- following treatment flies were raised on sterile food and tested on sterile food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 9-days post sleep deprivation, in CS-untreated, CS-mock treated and CS-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was higher in germ free flies but not significantly different to mock treated flies. (D) Average sleep bout duration during lights off (night bout) was significantly reduced in treated flies compared to untreated flies but was not different among mock and dc flies.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

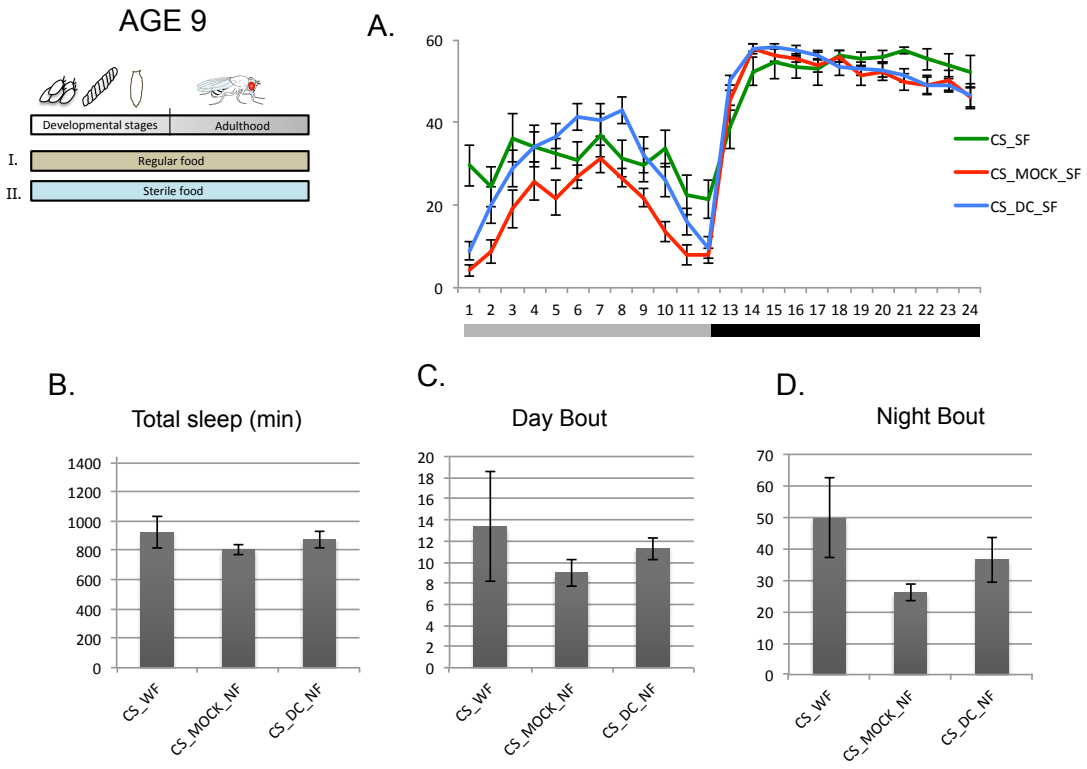

Figure 28. Sleep profile of sleep-deprived Canton-S flies (W-) post mock and axenization treatment- following treatment flies were raised on normal food and tested on normal food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 9-days in CS-untreated, CS-mock treated and CS-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies (C) Average sleep bout duration during lights on (day bout) was not significantly different between mock and dc flies, however both groups were significantly reduced compared to untreated siblings. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies, however both groups were significantly reduced compared to untreated siblings.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

4.5. Sleep profile of axenic flies upon starvation

In humans sleep loss is shown to induce hunger, whereas food deprivation suppresses sleep (MacFadyen et al. 1973; Pejovic et al. 2010). *Drosophila* displays a similar response upon starvation, and leads to sleep suppression (Keene et al. 2010). As mentioned in the introduction section microbiome composition depends on various factors, such as age, genetics and diet. Studies in fish and mice suggests that starvation leads to significant changes in the microbial composition of the gut (Okada et al. 2013; Xia et al. 2014). *Drosophila* microbiome dynamics upon starvation have not yet been studied. However considering the diet mediated shifts (Sharon et al. 2010; Chandler et al. 2011) it is highly possible that starvation leads to severe shifts in bacterial composition.

I analyzed the sleep behavior of axenic flies upon starvation, to investigate whether lack of microbiome impacts starvation induced sleep suppression. No difference in sleep suppression was observed between axenic and control flies.

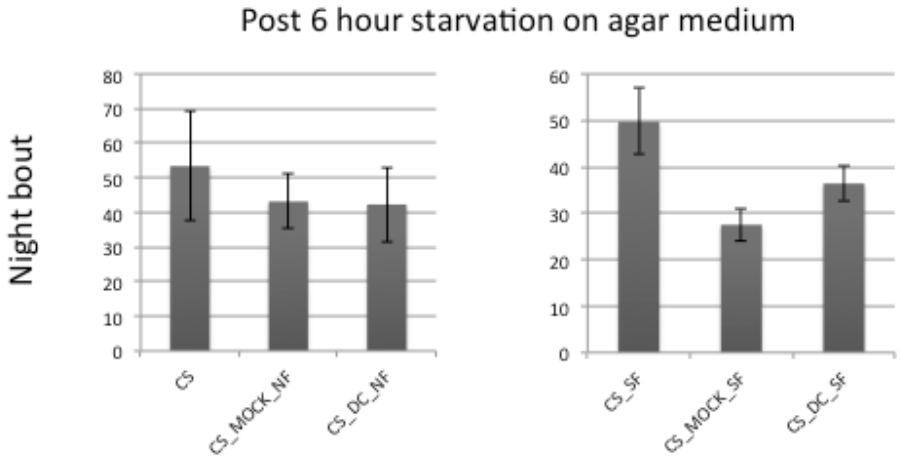

Figure 29. The analysis of starvation-induced sleep in axenic flies

There was no significant difference in the starvation-induced sleep behavior of mock treated and axenic flies. However both treated conditions displayed a reduction in night bout compared to untreated CS on normal and sterile food (4 day old female flies, n= 8 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

4.6. Sleep profile of axenic immune deficient flies

We used 2 different immune deficient flies; IMD^{1} and Rel^{E23} and asked whether early age microbiome loss alters their adult sleep behavior. The sleep profile of these flies are in agreement with the previously published data (Williams et al. 2007). These mutants were shown to have a 10-fold increase in the number of indigenous bacteria (Erkosar Combe et al. 2014). Therefore we were interested whether we could partially rescue the deficits in their sleep behavior by significantly reducing their bacterial number in their gut.

Untreated IMD mutants displayed a decrease in total sleep time compared to CS(W-). Whereas untreated RelE23 had an increased sleep compared to both wild type and IMD mutants. Day bout was decreased in IMD mutants and was increased in Relish mutants similarly night bout was decreased in IMD mutants and was increased in Relish mutants.

Immune deficient mutants were axenized with the same protocol, which was applied to CS flies. Interestingly dechorionation led to mortality in Relish mutants. Only few could survive until second instar larva stage, with most of them dying at embryonic stages. I tried using a less harsh protocol, by reducing the concentration of the used bleach, however this didn't reduce the mortality rate. IMD mutants also displayed a reduction in survival to adulthood, and a significant delay in development time.

At four days of age axenic IMD flies kept on regular sterile food showed similar sleeping behavior as their non-dechorionated siblings Interestingly, they displayed a significant decrease in daybout compared to the control mock treated group, which persisted at age 5 as well ($p=0.05$ student's t-test).

The axenic IMD flies, raised on non-sterile food had similar total sleep time compared to both their mock treated and untreated siblings. Interestingly there was a slight decrease in daytime sleep between treated group and untreated group, however day-bout between treated groups was similar. The night-bout in mock treated group was increased but it was not significantly different from untreated and axenic siblings.

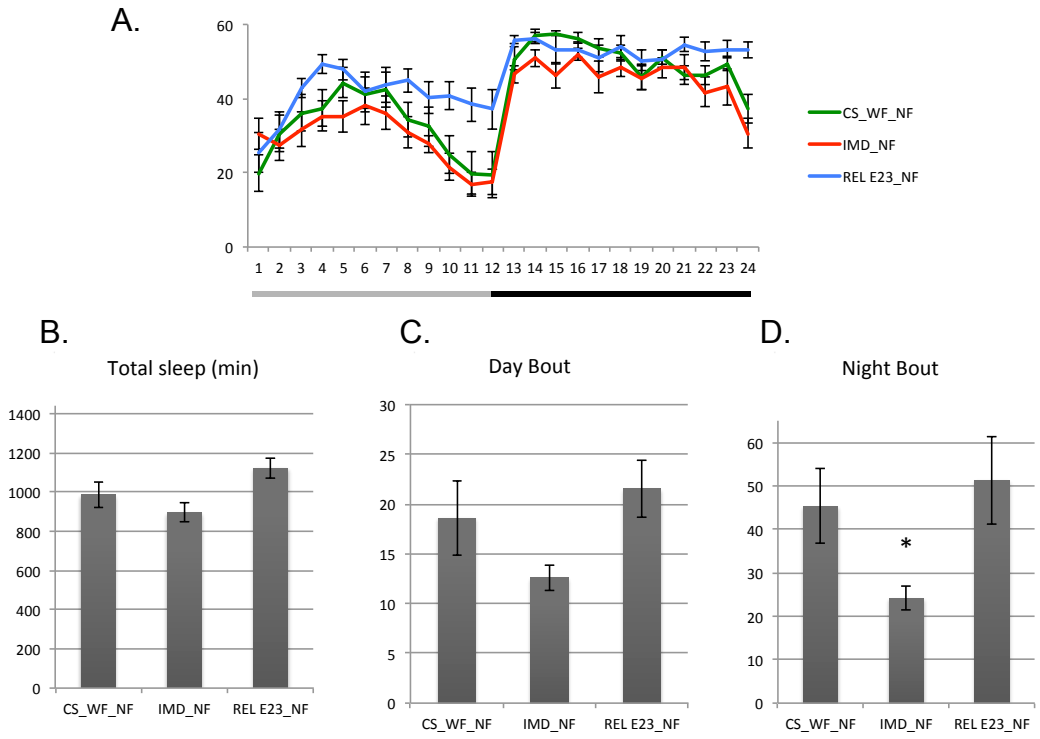

Figure 30. Sleep profile of Canton-S flies (W-), IMD^{1} mutants and Relish mutants

(A) Daily sleep in min/hour at age 4-days in CS(W-), IMD and Relish mutants. IMD mutants displayed a decrease in total sleep time compared to CS(W-). Whereas RelE23 had an increased sleep compared to both wild-type and IMD mutants Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes showed a decrease in IMD mutans and a decrease in relish mutants. (C) Average sleep bout duration during lights on (day bout) was was decreased in IMD mutants and was increased in Rel mutants (D) Average sleep bout duration during lights off (night bout) was similar to daybout profile and was decreased in IMD mutants and was increased in Rel mutants.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

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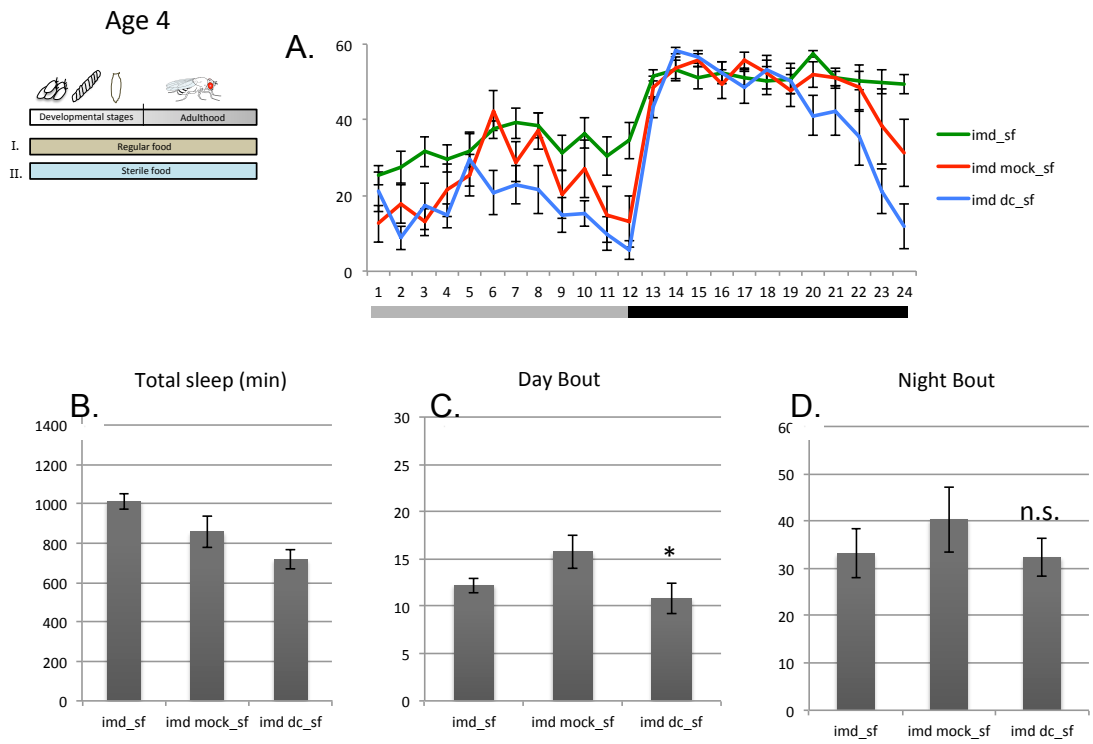

Figure 31. Sleep profile of IMD mutants post mock and axenization treatment- following treatment flies were raised on sterile food and tested on sterile food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in IMD-untreated (raised on sterile food), IMD-mock treated and IMD-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was significantly different between mock and dc flies.(p=0.04, student's t-test) (D) Average sleep bout duration during lights off (night bout) was decreased in germ-free IMD mutants not significantly different between mock and dc flies.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

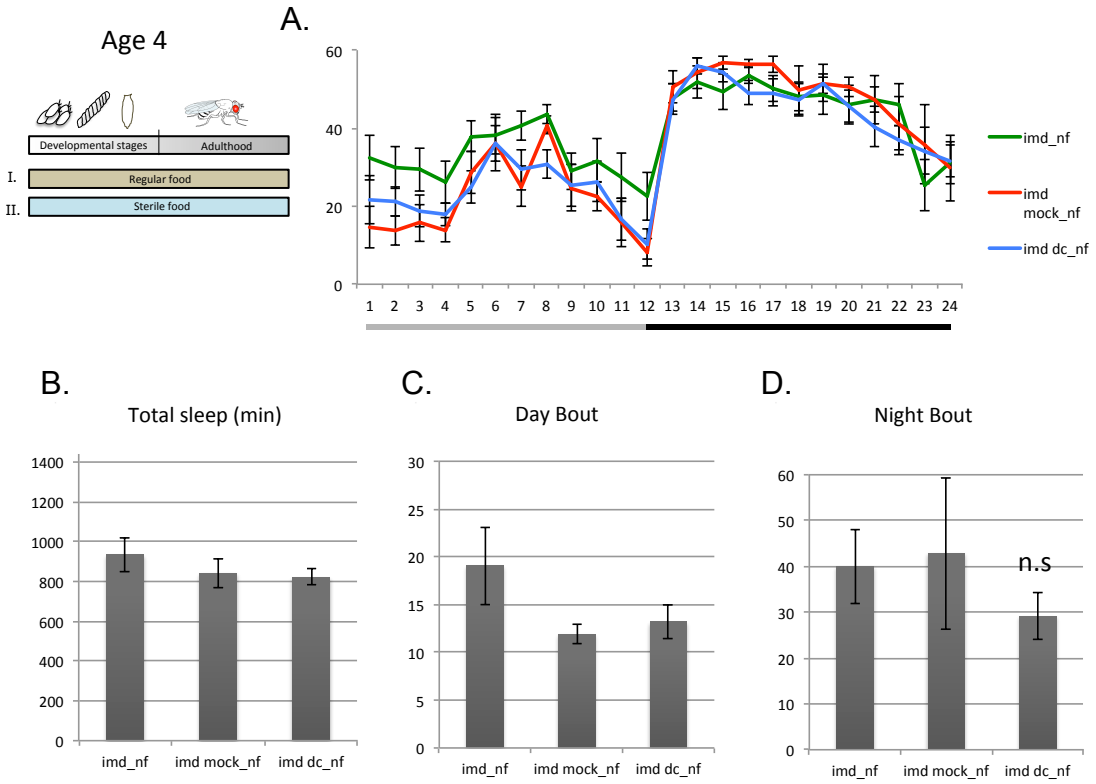

Figure 32. Sleep profile of IMD mutants post mock and axenization treatment- following treatment flies were raised on regular food and tested on regular food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in IMD untreated on regular food, IMD-mock treated and IMD-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was not significantly different between mock and dc flies. (D) Average sleep bout duration during lights off (night bout) was decreased in germ-free IMD mutants but was not significantly different between mock and dc flies.

(4 day old female flies, n=16 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

These data suggest that germ free IMD flies, raised under germ-free conditions have no drastic changes in their sleep as adults. Germ free W(-) IMD flies raised on non-sterile regular food, as well as germ free W(-) IMD flies raised on sterile regular food have similar total sleep time compared to their mock treated siblings. Dc flies on sterile food had a slightly significant decrease in day bout compared to mock treated siblings, however it was not significantly different from the untreated controls.

The sleep profile on regular food presented a similar profile. Interestingly there was again a treatment dependent decrease in total sleep time and also in day bout. However these were not significantly different among treated siblings. No obvious defects were prenst in night bout.

Taken together, similar to the observation among wild-type CS flies, no obvious microbiome dependent sleep deficits were present in immune deficient flies. However we didn’t analyze their sleep at old ages.

4.7. Supplementary results

AD, microbiome and sleep

We asked whether an Alzheimer's disease model in *Drosophila* might be influenced by the elimination of the microbiome. We used Gal4/UAS system to express the transgenic Alzheimer's constructs. The UAS APP; BACE construct was driven by the GR23 gal4. These flies had no obvious locomotor defects. However, these are reported to be age dependent and due to time constraints I couldn't evaluate their old age behavior.

I analyzed the sleep behavior of these flies at 5 days of age. I used CS (W+) and UAS APP; BACE/+ as genetic background controls. Interestingly one of the genetic controls UAS APP; BACE/+ showed significant changes in sleep behavior upon dechorionation, which was only present with flies raised on non-sterile normal food.

Similar to our observation in wild-type CS flies and IMD mutants no differences were observed in AD model flies upon microbiome elimination. However as mentioned before AD phenotype gets more severe in older flies and due to time constraints we couldn't evaluate the old age sleep profile of these flies.

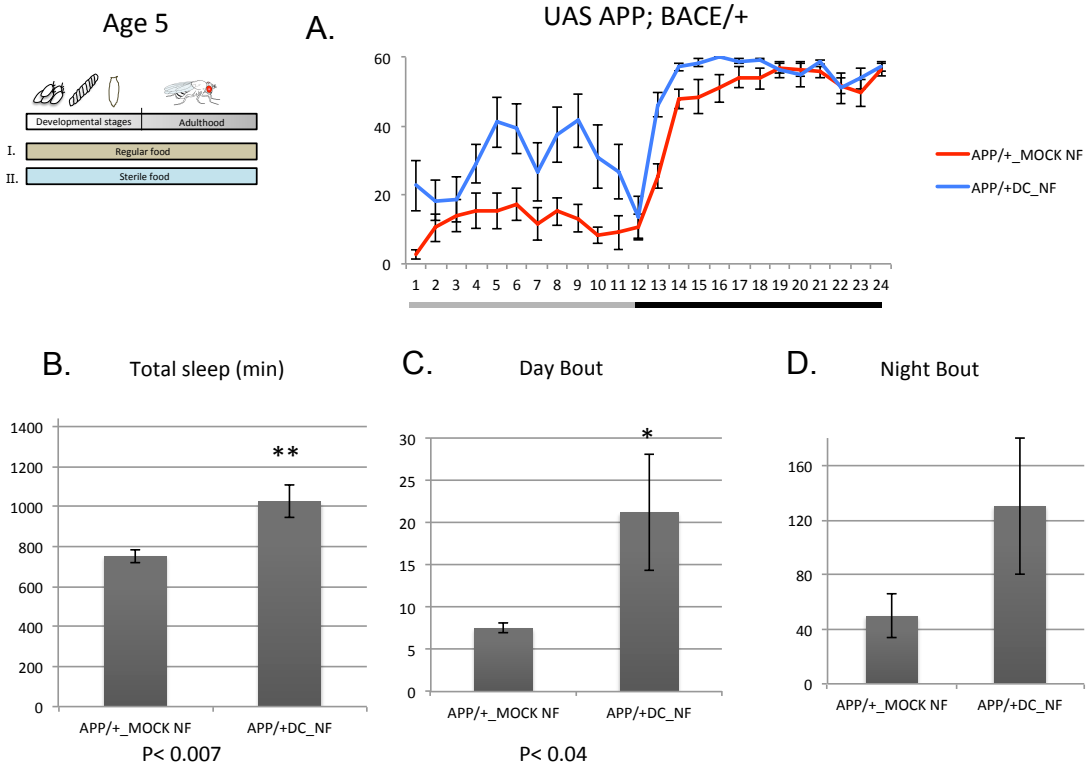

Figure 33. Sleep profile of genetic background control of AD flies, post mock and axenization treatment- following treatment flies were raised on normal food and tested on normal food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in APP;BACE/+ mock treated and APP;BACE/+ -dechorionated(dc). Light box indicated lights on and black box indicated lights off. (p=0.007, student's t-test) (B) Total daily sleep in minutes was significantly different in dc flies p=0.04 (C) Average sleep bout duration during lights on (day bout) was significantly reduced in dc flies compared to mock treated flies. (p=0.04, student's t-test)(D) Average sleep bout duration during lights off (night bout) was significantly increased in dc flies.

(5 day old female flies, n=8 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

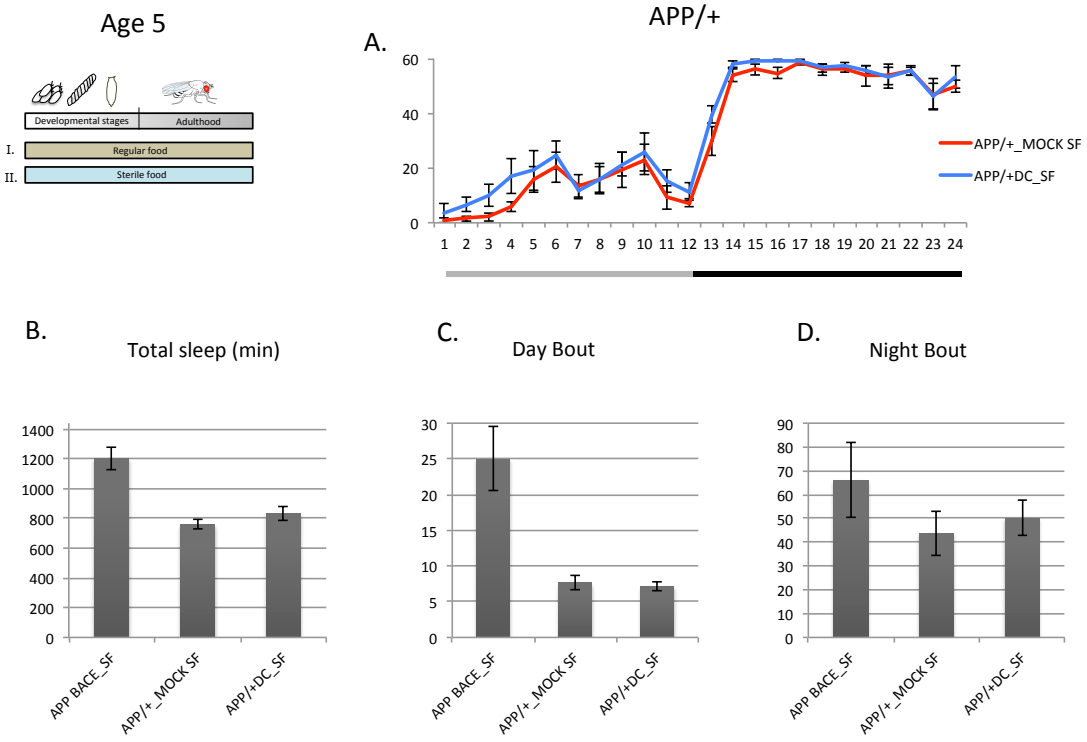

Figure 33. Sleep profile of genetic background control of AD flies, post mock and axenization treatment- following treatment flies were raised on sterile food and tested on sterile food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in APP;BACE/+ mock treated and APP;BACE/+ -dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was reduced in both treated group of flies, but was not significantly different between dc flies and mock treated flies. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies.

(5 day old female flies, n=8 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

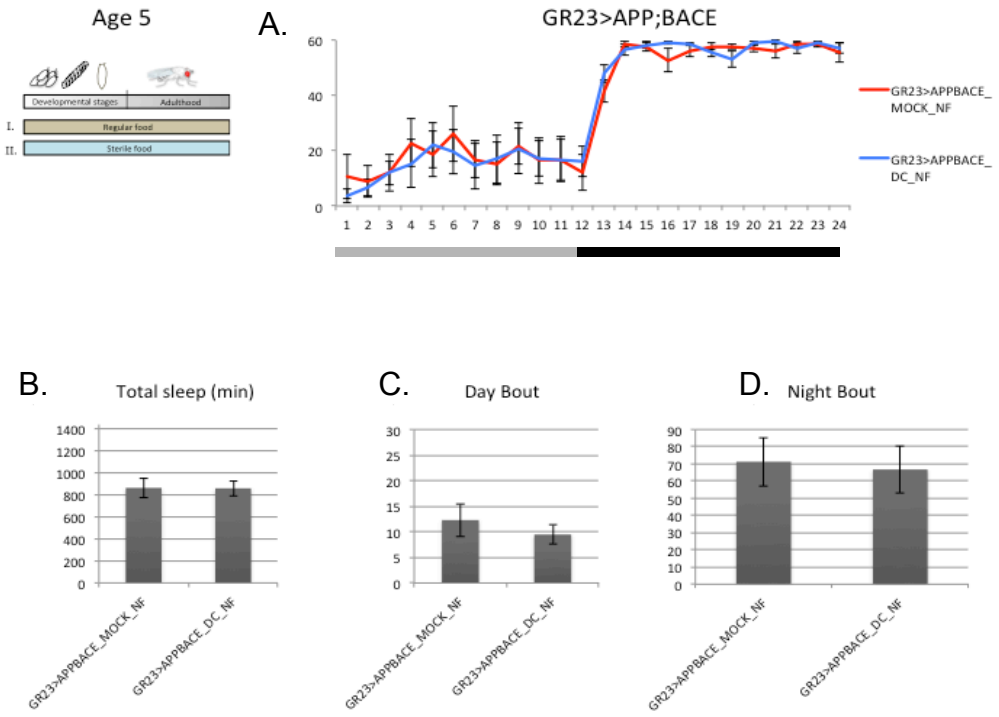

Figure 33. Sleep profile of AD flies post mock and axenization treatment- following treatment flies were raised on normal food and tested on normal food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in GR23>APP;BACE mock treated and GR23>APP;BACE-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was reduced in dc flies but is not significantly different to mock treated flies. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies.

(5 day old female flies, n=8 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

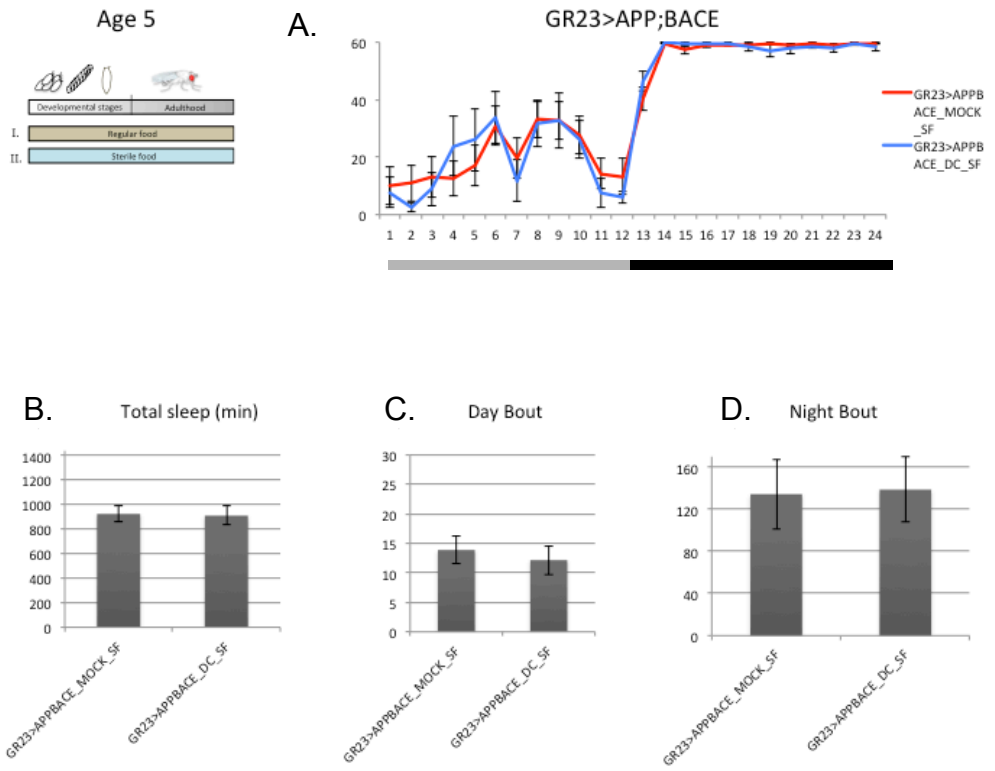

Figure 34. Sleep profile of AD flies post mock and axenization treatment- following treatment flies were raised on sterile food and tested on sterile food.

Sleep time was not significantly different between flies, which received the mock treatment and dechorionated (germ-free) siblings.

(A) Daily sleep in min/hour at age 4-days in GR23>APP;BACE mock treated and GR23>APP;BACE-dechorionated (dc). Light box indicated lights on and black box indicated lights off. (B) Total daily sleep in minutes was not significantly different between mock and dc flies. (C) Average sleep bout duration during lights on (day bout) was reduced in dc flies but is not significantly different to mock treated flies. (D) Average sleep bout duration during lights off (night bout) was not significantly different between mock and dc flies.

(5 day old female flies, n=8 for each group, n.s.= p> 0.05, Error bars represent s.e.m.)

Development and microbioime

In laboratory conditions (a temperature of 25°C, 50-60 %humidity with 12:12 light dark cycle) *D. Melanogaster* has a generation time of 10 days (Figure). After egg-laying, embryo follows a strict developmental program and after approximately 18-24 hours larvae hatch from the egg shell. After 3 Larval stages, which last about 4 days, larvae moult into immobile pupae. Pupal stages span over 4 days, after which adult flies hatch from their pupal case. The development time window is shown to be strictly temperature dependent and shows a delay at lower temperatures and a slight acceleration at higher temperatures.

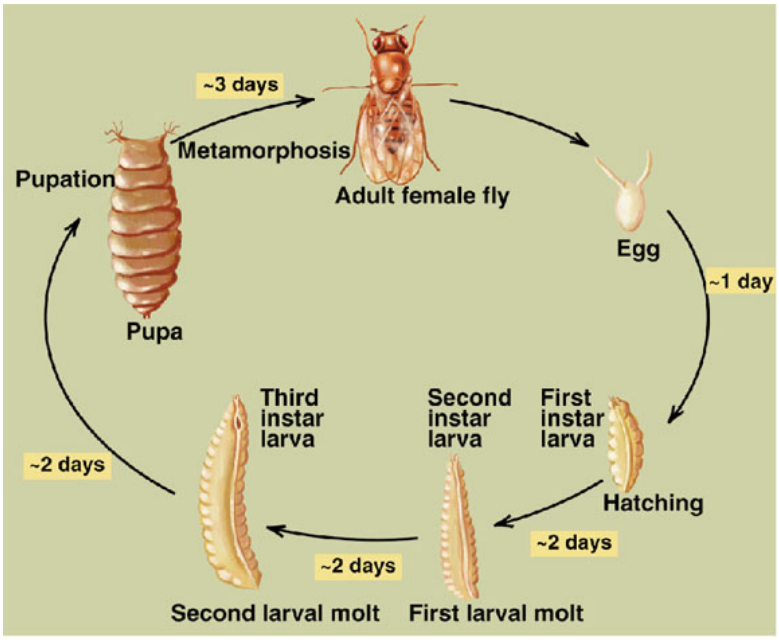

Figure 4. Life cycle of *D. Melanogaster*
(adapted from http://www.morgellonsuk.org.uk/micromyiasis.htm)
Drosophila life cycle spans over approximately 10 days. After egg- laying, embryonic development occurs at a rapid speed and larval hatching happens after 24h. Fruit flies larvae undergo 3 larval stages, with each stage spanning 2 days. After the last larval stage, third instar larva become immobile and pupariate for approximately 100 hours.

Interestingly during my project flies raised on sterilized food showed a delay in their development, although they were raised under the same conditions. Moreover the hatch rate in those vials was notably lower than the eggs transferred to normal food. No differences in adult body size were observed between these two groups.

These results could be due to several factors, which need further investigation. One problem with sterilized food was that it dried more often than the normal food. In order to prevent larvae from drying out, I added sterilized water to those vials, which might have affected the developmental program.

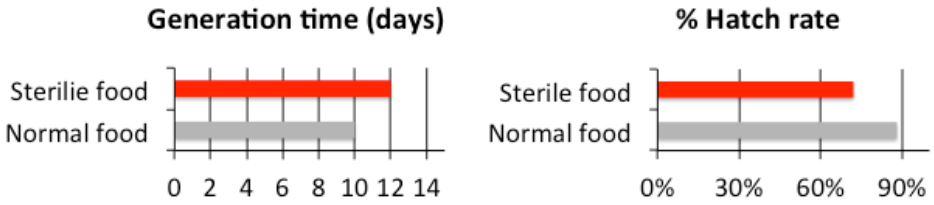

Figure 36. Flies raised on sterile food show a developmental delay

Eggs collected from CS flies were transferred to either sterile or normal food and were raised under same conditions.

5. Discussion

Is *Drosophila* a good model system to investigate sleep and microbiome link?

Sleep in *Drosophila* was discovered ~16 years by two different laboratories (Shaw et al. 2000; Hendricks, Finn, et al. 2000). Fruit flies met all the criteria of sleep; (1) They presented prolonged periods of quiescence, (2) They has reduced responsiveness' to external stimuli, (3) The state of sleep was rapidly reversible, (4) they presented increased need for sleep upon sleep deprivation, thus sleep homeostasis. Following this discovery, *Drosophila* has been employed as a model system for investigating the mechanisms of sleep.

The fruit fly as a model system offers numerous advantages for studying sleep; e.g. the ease for measuring sleep, screening for mutants and various behavioral assays. Drosophila genetics allowed manipulation of specific neuronal circuits. Therefore studying sleep in the fruit fly opened up a new way to address the sleep related questions, extending research beyond sleep deprivation studies. Sleep deprivation studies revealed numerous consequences of sleep loss, however not how these impact the animal's well being.

Nonetheless there are few drawbacks of fly sleep research. The obvious difference to consider is the neuroanatomical differences. Another important point to note is the neurochemistry; no noradrenaline, hypocretin/ orexin homologues are present in the fly. Lastly no REM-like phase has been identified in the fly so far. Yet the findings in *Drosophila* established important connections to sleep in humans. One such example is the study, which demonstrated that the increase in the levels of salivary amylase is common to both sleep deprived humans and flies.(Seugnet et al. 2006). This finding is particularly important, as it enabled a new noninvasive biomarker for sleep.

More importantly sleep is a phenomenon, regulated by and regulating multiple other functions. Sleep is obviously a unique behavior that is essential for a good health. Drosophila offers a unique system where one can easily combine genetics with a number of behavioral assays, which will allow the assessment of the functional outcomes of sleep alterations.

Main focus regarding sleep function has been on learning and memory (Donlea et al. 2011; Seugnet et al. 2008; Seugnet et al. 2009) and correspondingly on synaptic plasticity.

First studies on the fly microbiome were done almost 50 years ago, yet compared to sleep research, microbiome research in *Drosophila* is relatively immature(Bakula 1969). Most important advantage of the fruit fly microbiome is its relatively simple composition compared to mouse microbiome: both in number and genera. Up until recently mouse models were the models system of choice for microbiome studies. They have been extensively studied and several studies highlighted the importance of gut bacteria for the animal's well being. Dysbiosis of the microbiome is being implicated in several neurological diseases, ranging from mood disorders such as anxiety and depression to less understood complex disorders such as autism and Alzheimer's disease. Therefore considering the complexity and the multidimensionality of the microbiome, the need for simpler model is evident.

Fruit fly microbiome consists of 5-20 species. Similar to human microbiome, its composition and volume is age, diet and environment dependent. Under laboratory conditions flies are kept in vials on a specific diet. Therefore tracking the changes in microbiota resulting from diet is relatively easy.

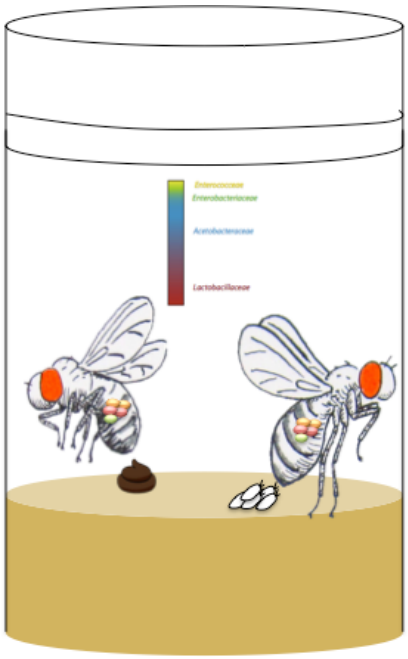

Figure 37. Fly microbiome under laboratory conditions
Under laboratory conditions flies are kept in vials. Initially drosophila embryos are sterile. However they quickly get associated with the bacteria from the food and from the feces of the adult flies. The core microbiome of *Drosophila* consists of Firmicutes, Bacteriodetes, Actinobacteria and Proteobacteria.

Until recently approaches for fly microbiome analysis were all invasive methods and flies had to be sacrificed. However a new non-invasive method has been recently published (Fink et al. 2013), which will allow the assessment of microbiota dynamics across lifespan.

My initial hypothesis was that development of a healthy microbiome is required for a healthy adult sleep behavior (Figure 38). My hypothesis based on two previously reported observations. First one is the study, which reported an increase in Anti microbial peptides (AMPs) upon sleep deprivation(Dissel, Seugnet, et al. 2015). *Metchnikowin (Mtk)* and *drosocin (dro)* mRNA transcript levels were significantly increased in sleep deprived animals. *Dro* showed an increase in neurons, whereas Mtk only in glia.These changes provided a possible explanation for the individual differences in resilience/vulnerability to sleep loss. Second observation was that the gut dysbiosis dependent fluctuations in AMP levels,(Lee & Hase 2014). Therefore it was intriguing to suggest that gut bacteria might be the key factors, which modulate responses to sleep loss by changing levels of certain immune factors.

Unfortunately my observations did not completely fulfill my hypothesis. There were no obvious changes in sleep behavior upon early age microbiome loss.

We then asked the question whether loss of bacteria makes them more vulnerable under stressful conditions. However I didn't observe any significant changes with the sleeping pattern of germ free wild type flies under stressful conditions, as they showed no difference in starvation induced sleep behavior or in homeostatic sleep response upon sleep deprivation.

Similar to the observation in wild type flies, sleep analysis of immune deficient mutants with higher number of microbiome bacteria didn't reveal any obvious defects as well. Germ-free IMD mutants displayed similar sleeping pattern compared to their untreated control siblings. However we couldn't assess whether they were more susceptible to stressful conditions such as starvation or sleep deprivation

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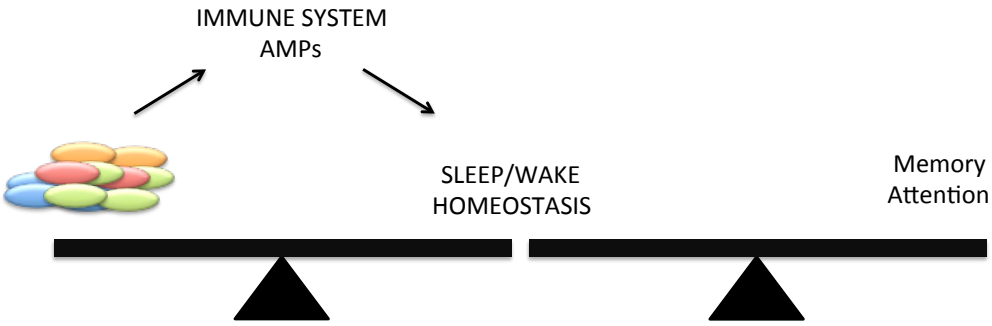

Figure 38. Initial Hypothesis part I

Immune sysyem is an important regulator of Sleep/ Wake homeostatis. My hypothesis suggested that a healthy composition of gut microbiome is essential to keep the immune signaling and thus the sleep homeostasis in balance. Considering the literature on the importance of healthy sleep on memory and learning, an equilibrium of all these factors is important for an animals well being.

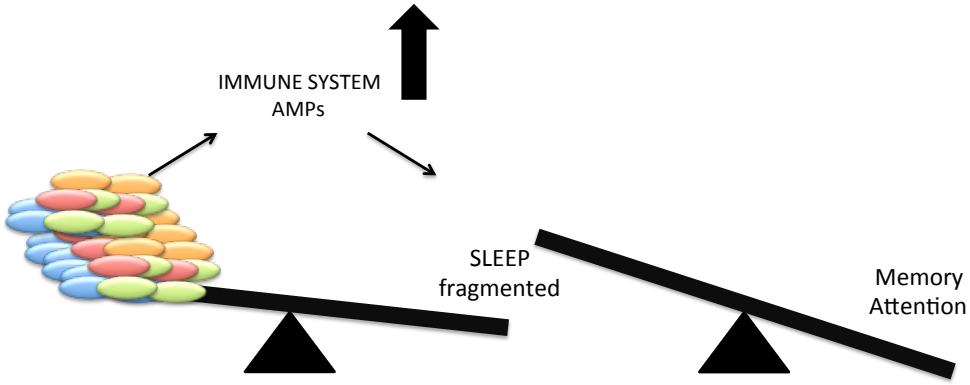

Figure 39. Initial Hypothesis- part Ii

Bacterial overload in the gut may result due to a number of factors; e.g. genetic susceptibility, as it is the case in immune deficient mutants or diet induced. This may explain the observed individual differences in AMP levels among sleep-deprived flies. however we cannot exclude whether these changes are initially a cause of sleep fragmentation or whether sleep fragmentation causes the changes in microbial levels.

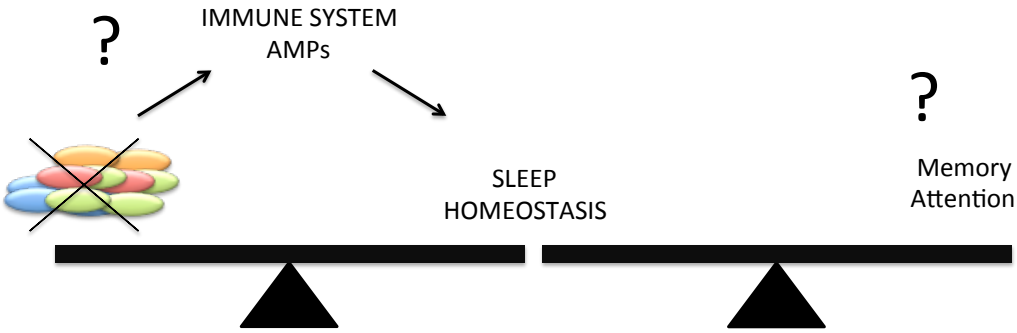

Figure 40. Initial Hypothesis- part Iii

What happens if we eliminate the bacteria in the gut ? we didn't observe any sleep related disturbances upon eliminating bacteria at early ages. We hypothesized that these flies would be more susceptible to stressful conditions. However these flies presented no obvious deficits upon two stressful conditions, which we tested.

The aging problem

One major stress factor, which I couldn't assess during my project, is "aging". Along with the physical changes that occur, aging is a common factor to sleep problems, microbiome dysbiosis and decline in brain plasticity. However we currently don't know whether these contribute to the process of aging.

Another arising question is whether we can use combinational therapy approaches to reduce the burden of aging on the brain, for example can we use sleep as a therapy for age related diseases? Or can we improve the quality of the microbiome in a way that it slows down the aging? And can these improvements restore the age dependent cognitive impairments?

One promising evidence came from a recent study, which demonstrated that sleep could restore memory to various *Drosophila* memory mutants, including models of Alzheimer's disease (Dissel et al. 2015).

As there is growing evidence for the connection between neurodegenerative diseases and microbiome (Shoemark & Allen 2014; Welling et al. 2015), is it possible to prevent or treat these age dependent diseases by changing the microbiome?

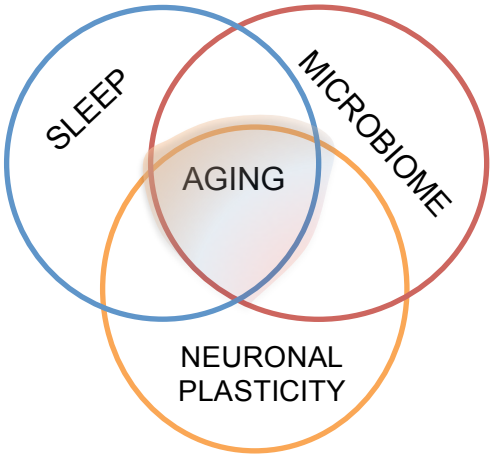

Figure 41. Sleep, Microbiome and Plasticity loop

All three of these show severe dysfunctions upon aging. One interesting question is whether and how these factors contribute to the course of aging. For example whether sleep loss during the early ages is a factor, which accelerates aging or whether one can slow down the course of aging by improving the low sleep quality at old ages.

Same questions can be asked for microbiome. As elderly show increased dysbiosis, can we improve life quality by bringing back the microbial homeostasis?

6. Future Directions

In our modern society microorganisms are almost always associated with diseases. There is a massive increase in the usage of germ-killing agents. Moreover the medical implication of antibiotics is rapidly increasing. It became a normal treatment option to prescribe antibiotics for even the basic flu. This not only led to a massive spread of antibiotic resistance but also to a shift in the microbial dynamics within our bodies. We are just starting to realize the importance of good bacteria and how they might be keeping a number of other systems within the body in balance.

Due to the emergence of faster and cheaper sequencing technologies microbiome research is in a booming era. It will be important to understand the basics of the bacteria-host interactions. Utilizing animal models, which allow the application of powerful genetics along with behavioral assays will provide a great basic understanding to these complicated mechanisms. These studies are essential to understand the complex interactions of human microbiome dynamics.

More importantly studying the consequences of these interactions and how they might be influencing or contributing to complex diseases, such as Alzheimer's disease will pave the way for new biomarkers for diseases and possibly to new therapy options. For example in the recent years there have been clinical studies, where an antibiotic, minocycline have been prescribed for treating depression and schizophrenia (Soczynska et al. 2012; Levkovitz et al. 2010; Liu et al. 2014). Another therapeutic approach gaining more interest is fecal microbiota transplantation (FMT). FMT is the transplantation of healthy fecal bacteria to a recipient, to restore the gut flora. It has been applied to treat *clostridium difficile* infection and many other gastrointestinal tract disorders. Recently FMT has been suggested as a therapy for neurological conditions (Borody et al. 2013). However apart from one case report, where FMT was observed to decrease Parkinson's symptoms, no other study in that regard has been published.

To sum up, understanding the microbiome, will not only allow a better understanding of our immune health, and susceptibility to conditions like

obesity, diabetes, and even depression but it will also pave the way for novel treatment approaches.

7. Acknowledgments

My four-month visit at Washington University in St. Louis was an excellent experience. WashU is located in St. Louis, and has two main campuses. My lab was located at the medical school campus, in the McDonnell sciences building. It's a great location, close to the metro station and offers great shopping and dining opportunities.

WashU medical school is ranked as one of the best medical schools in the United States and in the world. It was a great feeling to be among great scientists, who were ready to discuss science and share ideas even during short coffee breaks. Moreover I had the chance to attend excellent lectures and talks.

During my first week at WashU, I had to attend an orientation, where they informed us about everything we would need during our stay. Moreover staff at the international office was really friendly and were ready to help with any problem. The international office at the medical school was equally helpful. They guided me through my visa application and made sure that everything was submitted in time.

Last but not least Dr. Shaw and members of the Shaw lab provided excellent guidance throughout my project and also made sure that I had a great time outside the lab.

My internship enabled me to gain a great knowledge about sleep research and the techniques, which are currently used in the field. My future studies will build on the experience and knowledge, which I have acquired at WashU in Shaw Lab.

I would highly recommend WashU for future students, who would like to gain a great research experience in a small friendly and affordable mid-western city.

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