Interactions Between Sleep, Macrophages and Gut Microbiota of *Drosophila melanogaster*

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### Approval of Thesis

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Chapter III

Figure 1: Experimental Design for Antibiotic Treatment during development and adult stage.
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**Figure 2: Antibiotic treatment during development lead to decreased sleep behavior in adult flies.**

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (gray square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day. Sleep is shown as the average of sleep per 30 minutes of flies that have been treated with an antibiotic cocktail during developmental stages vs. control. Treated flies sleep less than flies that have not been exposed to antibiotics during a 24-hour day (mean ± SEM, n=47 control flies and 51 antibiotic treated flies). Two-way repeated-measure ANOVA identified a significant change in time x antibiotic treatment ($F_{[47,4512]} = 8.246, p < 0.0001$). (B) Effect of antibiotic exposure on total sleep during the night and day. White bars indicate control flies and gray bars indicate antibiotic treated flies. Total sleep is represented as the average of sleep in 12 hours. Flies exposed to the antibiotics show to have less total sleep during the night as well as during the day. Asterisk (*) indicates statistical significance. Repeated-measure ANOVA showed a significant change in the night ($p=0.0064$), day ($p<0.0001$) as well as interaction ($F_{[1.96]}=5.872$, $p=0.0173$). (Page 48)

**Figure 3: Antibiotics during development lead to decreased sleep duration and hyperactivity.**

(A) Sleep latency after lights off (night) and after lights on (day) indicates the average of minutes for control flies (white bars) and treated flies (gray bars) to demonstrate sleep on-set. No significant changes were observed when comparing control flies to those treated with the antibiotics. (B) Max sleep episode duration of flies during the night and the day. Flies treated (gray bars) showed a reduction in the duration of sleep bouts during the night ($p=0.0024$) as well as in the day ($p<0.0001$). Asterisk (*) indicates statistical significance. (C) Number of sleep episodes during the night and the day. Antibiotic treated flies showed a significant increase in number of sleep episodes ($p=0.0317$) when compared to non-treated flies. No changes were observed in the number of sleep episodes in the day. (D) Mean locomotive activity of flies during the waking period during the night and day. The treated flies showed a significant increase in activity during the night ($p<0.0001$) and during the day ($p<0.0001$) with significant interaction between night and day ($F_{[1.96]}=11.75$, $p=0.0009$). (Page 49)

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(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (gray square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day.
Sleep is shown as the average of sleep per 30 minutes of flies that have been treated with an antibiotic cocktail during adult stages vs. control. Adult flies that have been treated with an antibiotic cocktail sleep more than flies that have not been exposed to antibiotics during a 24 hour day (mean ± SEM, n=47 flies in control and 43 flies in antibiotic treated group). Two-way repeated-measure ANOVA identified a significant change in time x antibiotic treatment ($F_{[47,4136]} = 3.432, p < 0.0001$). (B) Effect of antibiotic exposure on total sleep during the night and day. White bars indicate control flies and gray bars indicate antibiotic treated flies. Total sleep is represented as the average of sleep in 12 hours. Repeated measure ANOVA shows that the adult flies exposed to the antibiotics have a total sleep significantly higher during the day ($p < 0.0001$) with no significant difference in total sleep during the night. There is a significant interaction between night and day ($F_{[1,88]} = 9.076, p= 0.0034$). Asterisk (*) indicates significance. (Page 50)

**Figure 5: Antibiotics during the adult stage lead to decreased sleep latency and increase in sleep duration.**

(A) Sleep latency after lights off (night) and after lights on (day) indicates the average of minutes for sleep on set in control flies (white bars) and treated flies (gray bars). Treated flies have a decrease sleep latency in the day ($p<0.0001$) with no change observed during the night. There is significant interaction observed between night and day ($F_{[1,88]} = 29.81, p<0.0001$). Asterisk (*) indicates significance. (B) Max sleep episode duration of flies during the night and the day. Exposed flies showed longer sleep episodes during the day ($p = 0.0003$) with no changes in the night’s episode duration. A significant interaction was observed from night to day ($F_{[1,88]} = 4.065, p=0.0468$). (C) Number of sleep episodes during the night and the day show no significant changes (D) Mean locomotive activity of flies during the waking period during the night and day show only a significant decrease during the day ($p=0.0224$). (Page 51)

**Figure 6: Combining development and adult stage antibiotic treatment leads to decreased sleep behavior.**

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (black square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day. Sleep is shown as the average of sleep per 30 minutes of flies that have been treated with an antibiotic cocktail during development through adult stage vs. control. Treated flies show to sleep less then flies that have not been exposed to antibiotics during a 24 hour day (mean ± SEM, n=47 flies in control and 50 flies in antibiotic treated group). Two-way repeated-measure ANOVA identified a significant change in time x antibiotic treatment ($F_{[47,4465]} = 9.340, p < 0.0001$). (B) Effect of antibiotic exposure on total sleep during the night and day. White bars indicate control flies and black bars indicate antibiotic treated flies. Total sleep is represented as the average of sleep in 12 hours. Flies treated with antibiotics sleep less during the night ($p=0.0334$) as well as during the day.
(p<0.0001) with an of interaction $F_{1.95} = 4.305, p= 0.0407$. Asterisk (*) indicates significance. (Page 52)

**Figure 7:** Sleep structure analysis reveals latency effects similar to adult treatment while sleep duration and activity effects resemble developmental antibiotic exposure.

(A) Sleep latency after lights off (night) and after lights on (day) indicates the average of minutes for sleep on set in control flies (white bars) and treated flies (black bars). Treated flies have lower sleep latency in the day (p<0.0001) with no significant change observed during the night. Interaction is observed between night and day ($F_{1.95} = 5.358, p=0.0228$). Asterisk (*) indicates significance. (B) Max sleep episode duration of flies during the night and the day. Treated flies show to have shorter sleep episodes during the night (p=0.0307) and during the day (p<0.0001). A significant interaction was observed from night to day ($F_{1.95} = 4.437, p=0.0378$). (C) No significant changes were observed in total number of sleep episodes neither in night nor day. (D) Mean locomotive activity of flies during the waking period during the night and day. Flies that were treated showed to have higher activating during the waking periods in the night (p<0.0001) as well as in the day (p=0.0094). Significant interaction between night and day was also observed ($F_{1.95} = 8.496, p=0.0044$). (Page 53)

**Figure 8:** In preliminary results, levels of synaptic protein tend to increase with antibiotic exposure.

(A) Western blot for detection of the synaptic protein DLG from Drosophila head lysate (5 heads per sample). Protein was detected with mouse anti-DLG, followed by IRDye 800 goat anti-mouse for control flies with no antibiotic exposure (Ctrl), for flies that received antibiotic exposure only during developmental stages (Dev), flies exposed as adults only (Adult) and flies that were maintained in the treatment all through development and as adults (Dev+Adult). B) Quantification of fluorescence intensity from western blot (A) (n=2). Analysis of one-way ANOVA showed no significance due to n value although, a tendency of an increase in DLG is seen in flies exposed to antibiotics. (Page 54)

**Chapter IV**

**Figure 1:** Experimental Procedure for the Generation of Flies with Eater Knockdown

Ten male UAS-Eater RNAi’s were crossed with ten virgin females with an Hml-Gal4 driver. The female Eater knockdown progeny was placed in the behavior monitors to record sleep behavior. (Page 69)

**Figure 2:** Eater knockdown flies leads to a decrease in total sleep during the night.

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (gray square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day.
Sleep is shown as the average of sleep per 30 minutes of flies with eater knockdown vs parental fly with functional phagocytic activity. Flies with impaired phagocytic activity sleep less during the night and seem to show a tendency to sleep slightly less during the day (mean ± SEM, n=121 flies in control and 121 flies in Eater RNAi). Two-way repeated-measure ANOVA identified a significant change in time x Eater RNAi (F_{47,11280} = 16.17, p < 0.0001). (B) Effect of impaired phagocytic activity on total sleep during the night and day. White bars indicate control flies and black bars indicate Eater RNAi flies. Total sleep is represented as the average of sleep in 12 hours. Repeated-measure ANOVA showed a significant change in total sleep during the night (p<0.0001) of flies with eater knockdown. Asterisk (*) indicates statistical significance. There is a significant interaction between night and day (F_{1,240}= 39.09, p<0.0001). (Page 70)

**Figure 3: Eater Knockdown results in shorter more frequent sleep episodes.**
(A) Sleep latency after lights off (night) and lights on (day) indicates the average of minutes for sleep onset in control flies (white bars) and Eater RNAi flies (black bars). Eater RNAi flies showed no significant change in the amount of time it took for sleep onset (latency) for the night or day (B) Max sleep episode duration of flies during the night and the day. Eater RNAi flies show shorter sleep episodes during the night (p = 0.0142) with no changes in sleep episode duration during the day. A significant interaction was observed from night to day (F_{1,240}= 7.201, p=0.0078). (C) Eater RNAi flies show an increase in sleep episodes during the night (p=0.0138) with no significant change during the day. (D) Mean locomotive activity of Eater RNAi flies are more active in their waking periods in the night and day (p<0.0001). (Page 71)

**Chapter V**

**Figure 1: Model for macrophage, gut microbes and sleep interaction**
(A) Proposed model for macrophage, gut microbes and sleep interaction during normal sleep. The circadian rhythm promotes higher phagocytic activity during the night of the gut microbiota producing in turn muramyl peptides (MPs) and cytokines that can induce sleep. (B) Proposed model for macrophage, gut microbes and sleep interaction during sleep deprivation. During sleep lost macrophages are activated by an unknown mechanism resulting in higher immune activity over the gut microbiota. This results in the release of sleep promoting MPs and cytokines by the activated macrophages and in this way play a role in sleep compensation after sleep loss. (Page 82)
List of Abbreviations

AMP – antimicrobial peptides
AXL – axenic locusts
BDNF – brain-derived neurotrophic factor
CFS – cerebrospinal fluid
CON – conventionally reared
ConL – conventionally reared locusts
CNS – central nervous system
DAMS – Drosophila monitoring system
DC – dendritic cell
DLG – discs-large
GF – germ free
GF+ – germ free mice inoculated with intestinal bacteria from conventionally reared mice
GF+p – progeny of the inoculated germ free mice
IEC – intestinal epithelial cells
IL-1 – interlukin-1
IL-6 – interlukon-6
LP – lamina propia
MP – muramyl peptides
NGFI-A – nerve growth factor-inducible clone A
OTU – operational taxonomic unit
POA – preoptic area
TLR – Tol-like receptors
TNF – tumor necrosis factor
QIIME – Quantitative Insights Into Microbial Ecology
Thesis Abstract

The study of the gut microbiota has taken great interest. With advances in technology of sequencing and computer analysis, it has opened great opportunities to study bacteria like never before. The presences of gut microbes have been associated with different aspects of development and behavior. With out a doubt, the intestinal microbiota is influencing more then just nutrition. In this work we focus our study in the role that the gut bacteria could be having on sleep and the function of the immune systems in this interaction. We aimed to answer the following questions: Is the gut microbiota affected by sleep behavior? Does the gut microbiota affect sleep behavior or patterns? Is the role of macrophage activity important in sleep behavior? To answer these questions we worked with *Drosophila melanogaster*, an important genetic tool for which the innate immune system and sleep behavior is well understood. We used next-generation sequencing of the 16s rRNA gene to identify the composition of the gut flora during the day and night as well as flies that have been sleep deprived compared to non-deprived. We also looked at the bacteria composition of flies that are not able to compensate for lost sleep (Pumilio knock out by RNAi). In these experiments we noticed a tendency to have a decrees in bacterial sequence reads during the night as well as in sleep deprived but not in flies that cannot compensate for sleep lost. We also notice a tendency of change in abundance certain bacteria groups such as *Alteromonadaceae* family. To focus on the effect of the gut microbes on sleep we treated flies with a broad-spectrum antibiotic cocktail during different developmental stages. Interestingly, flies treated with antibiotic during development show a decrease in total sleep while flies treated as adult show an increase in total sleep behavior. To elucidate if macrophage activity could be having an
important role in sleep regulation given that they are able to produce somnogenic substances as well as show more activity during the night, we used mutant flies that have impaired phagocytic activity to see the effect on sleep. We noticed that these flies show similar sleep structure as those flies that have been treated with antibiotics all during development, which show a decrease in total sleep. Taken together, the results seen in this work suggest that gut microbes, the immune system and sleep circuits interact to regulate sleep behavior under normal conditions, aside from that seen during an immune response against an infection. In addition, macrophage night activity seems to be playing a role in sleep regulation.
Author’s Biography

Yadira Ortiz Castellano was born in Caguas, Puerto Rico on April 1, 1980. She is the first born of Antonio Ortiz Colon and Carmen Sonia Castellano Castellano, followed by her brother, Eliu Ortiz Castellano born in 1981 and her sister, Bethzaida Ortiz Castellano, born in 1983. Yadira began her early education in Orocovies and Ciales, Puerto Rico. At the age of 6, her family decided to move to the state of Florida in search of better opportunities for their family. There she continued her education at Palmetto Elementary School, in West Palm Beach Florida and continued on to Conniston Middle School in the same city. There her geography teacher noticed her visual arts abilities and recommended a magnet school in which she could develop her talents. She was subsequently accepted in the visual arts program at Palm Beach School of Arts in which she studied for a year. Do to unplanned circumstances that required the family to move from Florida back to Puerto Rico, she was unable to pursue her education in the arts. For her senior year of High School she lived in Ciales, Puerto Rico at Juan Antonio Corretjer High School. After toying with the idea to study architecture or graphic arts, she decided to study Natural Science at the University of Puerto Rico at Cayey. Here she discovered the world of scientific research thanks to the RISE program. Although she did not continue her graduate education once she obtained her B.S. in 2004, it was always in the back of her mind. In 2012, the time was right to continue to pursue her dreams and was accepted into the Biology Masters Graduate Program at the University of Puerto Rico in Rio Piedras, which she completed in 2015. She wishes to further expand her knowledge and so will continue in Biology Doctorate Program at the same university.
Interactions Between Sleep, Macrophages and Gut Microbiota of Drosophila melanogaster
Dedicatory

This work is dedicated to the memory of my beloved grandfather:

Sixto Castellano Berdecia

May 6, 1925 to April 25, 2015.

He was an example of a loving husband, father, grandfather and great-grandfather. He was a hardworking honest man that always had a smile on his face, even in his illness. I will miss his smile, jokes and stories.

Te extrañare Abuelo.
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Chapter I

Background
Intestinal Microbiota

The use of high throughput sequencing has made it possible to study in more detail the population of microflora from all types of environments (Sleator, Shortall, & Hill, 2008). Previously, studies have been limited since 99% of bacteria cannot be cultured in the lab (Handelsman, 2004). It is with this tool that the field of metagenomics, the study of genetic material obtained directly from an environment, has taken off (Sleator et al., 2008). Now it is possible to identify the communities of the microflora, obtain large amounts of genetic information and study the effects that these could be having on the host. With all the data that is being collected, the use of bioinformatics is an important tool to manage, analyze and interpret this information by way of specialized computer software (Caporaso et al., 2010; Saeys, Inza, & Larranaga, 2007). Aside from its wide use in environmental studies, these scientific advances have also made it possible to study in depth the symbiotic relationship between host and microbes.

One of the largest symbiotic microbial flora is found in the intestinal track (Gill et al., 2006). In fact, it is now known that human intestinal flora outnumber somatic and germ cells ten fold (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; Savage, 1977). For years, it has been recognized that there is a mutualistic relationship between the gut flora and the host. These microorganisms are known to play an important role in supplying essential nutrients and aid in digestion (Zhang et al., 2015).

Establishment of the intestinal flora begins at birth. The composition is dependent of the first contact with the mother’s flora, feeding and the environment
(Fanaro, Chierici, Guerrini, & Vigi, 2003) although a study suggest that colonization of the gut may begin before birth and that it may come from the mothers mouths flora (Jimenez et al., 2008). However, studies have identified that the presence of gut microbes have an important influence over the development of the intestinal track's mucus layer, immune system and the central nervous system (CNS) (Deplancke & Gaskins, 2001; L Desbonnet, Clarke, Shanahan, Dinan, & Cryan, 2014; Diaz Heijtz et al., 2011; Kelly, King, & Aminov, 2007; Sudo et al., 2004). The use of germ-free animal models in various studies has shown that the presence of the gut flora is important for the maturation of the intestinal track. In mice, germ-free subjects show to have changes is epithelial cell structures (Bry, Falk, Midtvedt, & Gordon, 1996). For example, it has been found that there are fewer goblet cells, which are the specialized cells that produce mucus that lines the intestinal track (Deplancke & Gaskins, 2001; Kandori, Hiyama, Takeda, & Doi, 1996; Sharma, Schumacher, Ronaasen, & Coates, 1995). Also, the flora plays an important role in the development of gut immunity such as affecting Paneth cells, which secrete antimicrobial proteins (Cebra, 1999; Hooper, 2004; Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008).

The gut microbiota has also been found to influence the circadian clock of the intestinal epithelial cells (IECs)(Mukherji, Kobiita, Ye, & Chambon, 2013). In turn the composition of the microbes have been found to have daily oscillation that are affected by feeding as well as the molecular clock components of the host (Thaiss et al., 2014). Thaiss et al. (2014) discovered that disruption of the rhythm of food intake or circadian clock cause dysbiosis that have a metabolic effect.
Intestinal Microbiota and Behavior

It has also been observed that behavior of the host can be affected by the presence and or composition of the gut microbiota (Ezenwa, Gerardo, Inouye, Medina, & Xavier, 2012). The effect that the microbes have on behavior has been observed in insects such as locus swarming and mating of fruit flies and in mammals such as psychological implications observed in rats (Diaz Heijtz et al., 2011; Dillon, Vennard, & Charnley, 2000; K. M. Neufeld, Kang, Bienenstock, & Foster, 2011; Sharon et al., 2010).

The study of the desert locust and its gut microbes has found that the swarming of the desert locust is associated to a response of a pheromone that is found in faecal pellets (Dillon et al., 2000). Guaiacol, a key component of this pheromone, seems to be produced by the bacteria of the locust’s gut. This research involved the use of axenic locusts (AXL), which were reared by surface sterilizing the eggs and establishing a colony in sterile conditions. These axenic colonies were feed freeze-dried gamma radiated wheat. The control group was conventionally reared locusts (ConL) for which there were two groups: one group was fed the freeze-dried gamma radiated wheat, while the second group was fed fresh wheat seedlings which are part of their normal diet. When the fecal pellets were chemically analyzed, no guaiacol was detected in the samples obtained from the AXL colonies. Guaiacol was detected in both ConL colonies, however, there was a higher concentration found in the group fed the fresh wheat as oppose to the colony fed the freeze-dried wheat. These results suggest that the gut bacteria are
responsible for the production of guaiacol when it breaks down plant material on which the locust feed.

With *Drosophila melanogaster*, sex preference has been linked to the composition of the gut microbiota. Sharon *et al.* (2010) found that gut microbiota can influence the choice of the mating partners of the fly. In the study, the group observed that when two groups of the fruit flies were raised on different diets, molasses or starch based medium, there was a preference to mate with the fly that had been reared in the same type of food medium. To observe the mating behavior, a male and a female of each group were placed together in a mating chamber. Wings were clipped alternatively in each observation to identify the two variables.

Flies reared in a specific medium, molasses or starch, preferred to mate with other flies that were reared in the same medium. To confirm that this preference was influenced by the presence of distinct gut microbiota composition, flies were selected from each group and treated with an antibiotic cocktail (tetracycline, rifampicin and streptomycin), which was administered in the food. When the groups were observed in the mating chamber after antibiotic treatment, the preference was lost. These results suggest that bacteria, a particular species or specific composition of microflora, could be influencing the mating behavior.

With the use of germ free (GF) mice, a correlation between gut microbiota and social behavior has been observed (Diaz Heijtz *et al.*, 2011; K. M. Neufeld *et al.*, 2011). GF mice were compared to conventionally reared (CON) mice in test commonly used to measure their behavior, such as the light dark box test and the elevated maze test (an elevated equal length cross shaped stage, where two arms of
the cross are enclosed in walls and two are left opened). In the light dark box test the findings indicated that GF mice would spend more time in the lighted area and in the elevated maze test, they would spend more time in the open arm area of the maze. Other studies have shown that composition of the gut flora is also affecting behavior in mice. Bercik’s (2011) group compared two strains of mice, BALB and NIH SWISS (Bercik et al., 2011). BALB mice are known to display a very timid behavior; it is more hesitant to explore new areas. The NIH SWISS mice display a less timid behavior; they tend to venture more quickly to explore new areas. In this study, the two strains were treated with antibiotic to eliminate intestinal flora. The treated BALB mice were inoculated with microbiota of the cecal of the NIH SWISS strain while the treated NIH SWISS was inoculated with the microbiota of the cecal of BALB. When the behavior was observed now BALB mice which was described to be more timid showed a more adventurous behavior similar that of the NIH SWISS mice. The inoculated NIH SWISS mice was also affected and showed to be more hesitant to explore with the flora from the BALB mice. These results suggest that not only is the presence of the microbiota affecting behavior but that the composition of the bacterial community also plays a role.

The mechanism by which bacteria are able to manipulate behavior from the gut is not clear. However, the data that has been collected could indicate that these modulations could be occurring at a molecular level. Taking into account that the composition of the flora is a factor in the type of changes are observed in behavior suggest that bacterial products play an important role.
Intestinal Microbiota and the Central Nervous System

It is known that there is a bidirectional communication from the brain to the gut and vice-versa known as the brain-gut-axis (Bravo et al., 2011; Forsythe & Kunze, 2013). This communication is made up of various neuronal pathways of the autonomic neurons that connect the central nervous system to the esophagus, intestinal tract, liver and pancreas. Previously, studies had focused on the communication from the brain to gut; nonetheless the gut to brain aspect has been generating more interest. One of the neural pathways that could be of importance is the vagus nerve, one of the thirteen cranial nerves that originates in the medulla and extends to the chest and abdomen innervating the viscera. This nerve has been suggested to play an important role in establishing the communication from the intestinal microbes to the brain (Bravo et al., 2011; Forsythe, Sudo, Dinan, Taylor, & Bienenstock, 2010). Findings indicate that there is manipulation of gene expression in the brain (Bercik et al., 2011; Foster & McVey Neufeld, 2013). The communication seems to be aided by way of endocrine, metabolic and/or immune pathways by way of products derived from the bacterial population from the intestine. These bacterial products could be activating one or more of the pathways.

As before, the effects of the microbiome on the central nervous system (CNS) has been observed by the use of germ free animal models. In Diaz et al. (2011), GF mice showed a significant difference in anxiety like behavior when compared to CON mice. To conclude that the difference in behavior was due to the flora, the GF mice were inoculated with intestinal bacteria from CON mice (GF+).
The behavior test was carried out on the progeny of the inoculated GF mice (GF+p). The results showed that GF+p spent more time in the light, similar to GF mice but, spent less time in the open spaces in the open arm test, similar to CON. The test was repeated with the GF+ resulting in behavior similar to the GF mice. These could implicate that the microbiota is also exerting an effect over a point in early development that affects behavior in the adult stage.

For a better understanding of the changes that occurs at a molecular level, Diaz’s group (2011) measured the expression of genes, in particular nerve growth factor-inducible clone A (NGFI-A) and brain-derived neurotrophic factor (BDNF). These two proteins have been implicated in the development of anxiety. Lower expression of these genes was found in the frontal cortex, striatum, amygdala and the hippocampus of the GF mice by using in situ hybridization. Other research has also shown lower expression of these two genes in GF mice (Sudo et al., 2004). Similar results of lower levels of BDNF were found in the amygdala of mice treated with antibiotic for seven days (Bercik et al., 2011). As seen in the GF mice, the antibiotic treated animals presented more exploratory behavior in the lighted area of the light-dark box test and less apprehensive in a step down test. Changes in synaptogenesis have also been found in relation to gut microbiota. GF mice show to have higher expression of synaptic related protein, PSD-95 and synaptophysin in the striatum (Diaz Heijtz et al., 2011).

This research suggests that the microbiota is modulating, to a certain point, the expression to select genes. Aside from anxiety, depression, stress, schizophrenia, autism, and Alzheimer's disease have also been associated with a decrease in levels
of BDNF (Berry et al., 2012; Karege et al., 2002; Olsen, Kaas, Schwartz, Nykjaer, & Glerup, 2013; Phillips et al., 1991; Shimizu et al., 2003; Taurines et al., 2014; Toyooka et al., 2002). The conditions listed have also been associated to disturbances of the intestinal flora (Adams, Johansen, Powell, Quig, & Rubin, 2011; Bailey et al., 2011; Bailey, Lubach, & Coe, 2004; Finegold et al., 2010; Karri, Acosta-Martinez, & Coimbatore, 2010; Parracho, Bingham, Gibson, & McCartney, 2005). Meanwhile, sleep disturbances have also been associated with these conditions (Krakowiak, Goodlin-Jones, Hertz-Picciotto, Croen, & Hansen, 2008; Mayes, Calhoun, Bixler, & Vgontzas, 2009).

**Intestinal Microbiota and the Immune System**

The immune system has an important role in the gut microbiota homeostasis. There are immune mechanisms in place to control the exposure of intestinal bacteria to other areas of the body. One of these is a mucus layer secreted by goblet cells of the intestine. The mucus is composed of mucin glycoproteins that form one layer in the small intestine and two layers in the colon (Johansson, Larsson, & Hansson, 2011; Johansson, Ambort, et al., 2011; Lora V. Hooper, Dan R. Littman, & Andrew J. Macpherson, 2012). Paneth cells, another type of epithelia cell of the intestine, are able to secrete antimicrobial peptides by activation of Tol-like receptors (TLR) (Vaishnava et al., 2008). Another line of defense is the lamina propia (LP), which is a loose connective tissue that supports the mucosa layer. Here there are dendritic cells (DC) that will sample bacteria from the mucosa epithelium and present the antigen at the mesenteric lymph nodes for the production of IgA by the B-cells (A J Macpherson, Hunziker, McCoy, & Lamarre, 2001; Andrew J
Macpherson & Uhr, 2004). In the LP there are also macrophages that along with DC will also engulf any bacteria that manages to breach the epithelium (Kelsall, 2008).

With this immune activity, the presence of inflammation would be expected. A study by Rimoldi (Rimoldi et al., 2005) found that the release of inflammatory cytokines is dependent on the type of activation signal that activated the DC. It was found that the DC was activated by epithelia cell stimulated by a microbe; the cytokines produce will be non-inflammatory such as IL-10. However, if the DC has direct contact with intestinal bacteria from the lumen by penetrating a dendrite between the adjacent epithelium cell, will cause more of an inflammatory response with the release of IL-12 as well as IL-10.

**Sleep and the Immune System**

The function and regulation of sleep is a large field of study and it still holds many unanswered questions. There seems to be various important functions of sleep that have been specified to date as well as various mechanisms that influence the regulation of sleep. Sleep behavior has been associated with cognitive functions, clearance of metabolites from the brain and immune system activity among others (Gomez-Gonzalez et al., 2012; M. Irwin et al., 1994; McCoy & Strecker, 2011; Poe, Walsh, & Bjorness, 2010; Prince et al., 2013; Williams, Sathyanarayanan, Hendricks, & Sehgal, 2007; Xie et al., 2013). The regulation of sleep involves circadian oscillation of hormones, neurotransmitters and other sleep promoting factors in various pathways (Cirelli, 2009; Obal Jr. & Krueger, 2003). Components of the immune system, such as Interlukin-1 (Il-1) and Tumor necrosis
factor (TNF), have also been classified as sleep regulating substances (Kapsimalis et al., 2008; James M Krueger, Rector, & Churchill, 2007; James M Krueger, 2008).

Findings show that disruption in sleep alters the immune system and the response of the immune system to an infection and/or injury (M. R. Irwin et al., 2008; James M Krueger et al., 2007; Majde & Krueger, 2005; Mullington, Simpson, Meier-Ewert, & Haack, 2010; Takahashi, Kapás, Fang, & Krueger, 1999). If there is deprivation of sleep, there is a noted increase in the circulation of leukocytes as well as cytokines and inflammatory markers that can lead to health issues (Everson & Toth, 2000; M. R. Irwin, Carrillo, & Olmstead, 2010; Kuo, Pike, Beizaeipour, & Williams, 2010). What remains unclear is how sleep deprivation is activating an immune response.

In early studies, it was found that when cerebrospinal fluid (CFS) from sleep deprived goat was injected into rested rats, it provoked sleep (Pappenheimer, Miller, & Goodrich, 1967). Subsequent studies described the sleep inducing substance in CFS as muramyl peptides (MP) (J M Krueger, Pappenheimer, & Karnovsky, 1978, 1982; J. M. Krueger, Bacsik, & Garcia-Arraras, 1980). Studies also confirmed that muramyl peptide, which is derived from bacterial peptidoglycan, can provoke sleep in rested animals (J M Krueger et al., 1978). When chronically deprived, healthy rats developed a systemic infection with bacteria that had been associated with the intestinal microbiota (Everson & Toth, 2000).

Sleep deprivation also affects the effectiveness of certain aspects of the immune system. Circadian rhythm has been observed in the immune system (Bollinger et al., 2009; Cermakian et al., 2013; Keller et al., 2009; Kuo et al., 2010;
Narasimamurthy et al., 2012). Bollinger et al. (2009), for example, found the highest concentration of regulatory T cells in humans is at night although; the suppressive ability is affected if there is sleep deprivation. They also showed that the T cells are also affected by sleep deprivation showing lower concentrations the morning after. Proteins associated with the circadian clock have also revealed an effect over the immune response such as phagocytosis and cytokine expression (Cermakian et al., 2013; Narasimamurthy et al., 2012; Stone et al., 2012).

The gut microbiota is affected by and affects the immune system, central nervous system and intestinal epithelium cell circadian rhythm (Deplancke & Gaskins, 2001; L Desbonnet et al., 2014; Diaz Heijtz et al., 2011; Kelly et al., 2007; A J Macpherson et al., 2001; Andrew J Macpherson & Uhr, 2004; Mukherji et al., 2013; Sudo et al., 2004). Sleep is also affected by as well as affects the immune system, the central nervous system and intestinal homeostasis (Imeri, Bianchi, & Mancia, 1997; Kapsimalis et al., 2008; James M Krueger et al., 2007; James M Krueger, 2008; Silverman, Hong, & Karnovsky, 1985). However, to the best of our knowledge, a link between the gut microbiota and sleep behavior has not been studied. A better understanding of gut microbes interaction with sleep and the immune system could offer a better understanding of not only the role of the microbiota on the host, but also on health and lead to more effective medical treatments of sleep disturbances as well as intestinal diseases.
Chapter II

The Composition of *Drosophila melanogaster*’s Gut Microbiota in Relation to Sleep Behavior
Abstract

Despite mayor advantages in our knowledge of the neural circuits, genes and biological processes involved in sleep regulation, the homeostatic control of sleep behavior remains poorly understood. Early classical experiments transferring cerebrospinal fluid (CSF) from sleep-deprived animals into rested non-deprived animals established that the homeostatic control of sleep involved the accumulation of sleep-inducing substances. Later studies identified fragments of bacterial cell wall products called muramyl peptides (MPs) as important sleep-inducing substances. Although it was known that MPs could be produced by phagocytosis of gram-negative and gram-positive bacteria, whether the bacterial source came from opportunistic infections, endogenous bacteria or contamination has been the subject of a long-standing debate and speculation. Here we take advantage of the sequencing tools available in our genomic era to shine light into this issue. We show for the first time that: 1) the amount and composition of gut microbes changes between sleep and wake flies; 2) sleep deprivation leads to a global decrease in the number of gut microbes and alters the relative abundance of specific bacterial strains and; 3) sleep homeostasis is linked to the global reduction in gut microbes and an increase in bacteria from the Alteromonadaceae family. Taken together, these results indicate that gut microbes play a role in both normal sleep regulation and the response to sleep deprivation. Given the alarming increases in the prevalence of chronic sleep deprivation and related disorders in our society and the accessibility of gut microbes, our findings could provide the basis for both diagnostic tools for sleep conditions as well as novel treatments.
1. Introduction

Up until recent years, the focus of the studies of microbes was mostly focused on the effects of pathogenic microbes on the host. Even these studies have been limited since only 1% of the bacteria can be cultivated in the lab (Handelsman, 2004). With the development of high throughput sequencing, it has made it possible to study a wider range of bacteria and identify, microbial population from all types of environment (Sleator et al., 2008). With the use of specialized computer programs, the genetic material can be analyzed and interpreted, obtaining a wide range of information (Caporaso et al., 2010; Saeys et al., 2007). This technology has also made it possible to study the symbiotic relationship between the microbes and the host. The intestinal microbiota has been found to be the largest symbiotic community with ten times more bacteria than human cells (Backhed et al., 2005; Gill et al., 2006; Savage, 1977). It is well known that the gut microbes have a mutualistic relationship with the host providing essential nutrients as well as aid in digestion (Zhang et al., 2015), however, in recent years with the study using germ-free animal, it has been found that the presence of the gut microbes also has an effect on the immune system, gut development and the central nervous system gene expression (Deplancke & Gaskins, 2001; L Desbonnet et al., 2014; Diaz Heijtz et al., 2011; Kelly et al., 2007; Sudo et al., 2004). The presence of the gut microbes has also been linked to changes in behavior in insects and mammals (Diaz Heijtz et al., 2011; Dillon et al., 2000; Ezenwa et al., 2012; K. M. Neufeld et al., 2011; Sharon et al., 2010). Nonetheless, a link between sleep and the gut microbes has not been studied.

Early studies in sleep deprivation have identified a sleep inducing substance in the cerebral spinal fluid (CSF) that was later identified as muramyl peptide (MP)(J M
Krueger et al., 1978, 1982; J. M. Krueger et al., 1980; Pappenheimer et al., 1967). MP is derived from bacterial peptidoglycan, component of the cell wall which has been confirmed to induce sleep (J M Krueger et al., 1978). MPs are released as a process of the phagocytized bacteria by the macrophages as well as a byproduct of bacterial cell division (Johannsen, Wecke, Obál, & Krueger, 1991; Wheeler, Chevalier, Eberl, & Gomperts Boneca, 2014). In depth studies into the mechanisms of MPs on sleep has demonstrated that there are at least two possible pathways. An indirect pathway involves the immune system. MPs are produced and released during the digestion of bacteria by macrophages (Johannsen et al., 1991). The presences of MPs activate macrophages which in turn release sleep inducing pro-inflammatory cytokines such as Interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Dinarello & Krueger, 1986; Nosо, Parant, & Chedid, 1988; Pabst, Beranova-Giorgianni, & Krueger, 1999; Silverman et al., 1985). MPs also have a direct effect over the central nervous system sleep related networks such as GABA receptors (Grigoriev, Petrova, Gabreliyan, & Ivanova, 2008; Imeri et al., 1997).

Circadian rhythm has been observed in immune activity in insects and mammals(Cermakian et al., 2013; Curtis, Bellet, Sassone-Corsi, & O’Neill, 2014; Cutolo, Seriolo, Craviotto, Pizzorni, & Sullì, 2012; Keller et al., 2009; Kuo et al., 2010; Lee & Edery, 2008; Narasimamurthy et al., 2012; Scheiermann, Kunisaki, Frenette, Frenette Ruth L, & Gottesman, 2013; Stone et al., 2012). For example, in humans, the pro-inflammatory cytokine such as TNF and interleukin-6 have circulating peak at 3 am and 6 am. In the fruit fly, a circadian rhythm is observed with an increase in immune activity. Although in insects the humoral arm of the immune system (antimicrobial peptides
release) appear to lack circadian regulation, insect macrophages (plasmatocytes) exhibit robust oscillations that peak during the night (Lee & Edery, 2008; Stone et al., 2012). Together this information would suggest that the increase activity of macrophages during the night would contribute to the increase circulation of cytokines and MPs.

Although there is an understanding of the effects of the MP on the immune system and sleep, the origin of MPs found during sleep deprivation when there is no infection present is not clear. We theorize that these peptidoglycan fragments originate from the gut microbiota. It has been observed that rats that have suffered chronic sleep deprivation develop sepsis with the mesenteric lymph node infected with bacteria that have been associated with the intestine (Everson & Toth, 2000). This would suggest that sleep could play an important role in gut microbiota homeostasis. To elucidate if sleep has an effect over the composition of the gut microbiota, the gut of Drosophila melanogaster was dissected at different time points and after sleep deprivation.
2. Material and Methods

2.1 Fly Stocks

For the experiment looking at the day (ZT-5) and night (ZT-21) time points the wild type strain of *Drosophila melanogaster*, Canton S was used. Flies were raised at 25°C on banana-based medium (Heed’s banana medium). 2 to 3 day old female flies were used to carry out experiment.

For the study of the effects of sleep deprivation on the gut microbiota a cross of Tim-Gal4 and UAS-*PUM* RNAi was used. The control groups consisted of the first familial generation without the knockdown determined by the curly wing phenotype. The experimental group consisted of the first familial generation with pumilio knockdown determined by the straight wing flies.

2.2 Circadian Training

To monitor sleep behavior the *Drosophila* Monitoring System (DAMS) form Trikinetics was used as previously described (Chiu, Low, Pike, Yildirim, & Edery, 2010; Shaw, Cirelli, Greenspan, & Tononi, 2000). 2 to 3 day old female flies were used. The experimental and the control groups were placed in programmable incubators (Percival Model I-30 BLL) set with 12:12 light-dark cycle with a temperature of 25°C and at 80% humidity. One monitor was programed for light from 5:00 pm - 5:00 am while the other was programed for light from 11:00 am to 11:00 pm. Flies were removed from the incubator at two time points (at ZT-21 and ZT-5). The flies were quickly transferred to microtubes and placed in dry ice.
2.3 Sleep Assay

To monitor sleep behavior the Drosophila Monitoring System (DAMS) form Trikinetics was used as previously described (Chiu et al., 2010; Shaw et al., 2000). The experimental and the control groups were placed in a programmable incubator (Percival Model I-30BLL) set at a 12:12 light-dark cycle with a temperature of 25°C and at 80% humidity. After placing the flies in the monitors and incubator, they were allowed to adapt and normal sleep was allowed for a week. After a week, the flies were transferred to new tubes with fresh food and placed in the incubator for mechanical sleep deprivation.

2.4 Gut Dissection

Before dissecting flies, they were surface sterilized by a series of washes. The first step in the wash is in a 2.5% surface solution, followed by a quick wash in 70% ethanol and lastly in sterile Drosophila Ringer solution. All surfaces and instrument were cleaned with 70% ethanol. After the wash, flies were placed in Drosophila Ringer solution and the guts were dissected under a stereomicroscope. The dissection process was carried out over cold ice and the dissected gut was immediately placed in a cold sterile microtubes.

2.4 DNA Extractions and Sequencing

The dissected guts were placed in a Power Soil DNA Isolation Kit’s 96 well bead plate. The plates were shipped to Argonne National Laboratory for DNA extraction and sequencing. The V4 region of the 16s rRNA gene was amplified using the PCR primers FWD:GTGCCAGCMGCCGCGGTAA and REV:GGACTACHVGGGTWTCTAAT using Illumina MiSeq technology.
2.5 Bioinformatics Analysis

The sequencing data was analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). The sequences were aligned using green gene database. Rarefaction was applied to the operational taxonomic units (OTU) to calculate beta diversity. OTU significance was also calculated and analyzed in Prism.
3. Results

3.1 The amount and composition of gut microbes change as a function of time of day

To investigate the relationship of sleep and gut microbes, we compared the microbiota of fruit flies during a sleep time point in the night (ZT-21) vs. a wake time point during the day (ZT-5). Aside from the arousal differences, these time points were chosen because they represent the times of day of low (ZT-5) and high immunity (ZT-21) (Lee and Edery, 2008). Moreover, macrophages, which play a key role in both the processing of sleep inducing substances of bacterial origin and gut homeostasis, are more active during the night than during the day (Stone et al., 2012). Based on this evidence, we hypothesized that the overall number of gut microbes will be higher during the day than during the night and that their composition may also vary across the day. To test this hypothesis, we examined the sleep patterns of female wild type Canton-S flies, dissected the gut of one group at ZT-5 and another group at ZT-21, and examined the number and composition of their gut microbiota using 16S rRNA amplification and sequencing.

Consistent with the idea that nighttime phagocytosis of gut microbes induces sleep, we found that the night time point is associated with a reduction in the total number of sequence reads which suggest an overall decrease gut microbes (Figure 1A). In general the relative abundance of the different microbes was similar between sleep and wake flies at the selected time points (Figure 1B). However, ANOVA analysis in QIIME comparing each bacterial strain during the night and day time points revealed interesting tendencies in several strains. For example, a specific bacteria from the Sapropirae (class) and saprospiraceae family is increased in wake flies during the day while another strain from the Gammaprotobacteria class and Pseudomonas genus decreased (Figure 1C). Although
these results require further examination with independent methods, they suggest that gut microbes regulate sleep or vice versa.

3.2 Sleep deprivation reduce the number of gut microbes and alters the relative abundance of specific bacterial strains. We reasoned that if sleep plays a role in the regulation of gut microbes, then eliminating sleep using mechanical sleep deprivation should lead to changes in the number and composition of gut microbiota. To test this hypothesis, we chronically sleep deprived flies for 4 nights and 3 days and collected all samples in the first morning (ZT-2) after the deprivation period. Under these conditions the circadian time and the light-dark cycle, which could be confounding factors in our first experiment, are controlled since they are the same for the two groups. Since the immune system regulates gut microbes (You et al., 2014) and it is thought that the immune system is suppressed by sleep deprivation (Imeri and Opp., 2009), we predicted that sleep deprivation would be associated with higher diversity and number of gut microbes.

In contrast to our hypothesis, we found that sleep deprivation reduces the total number of gut microbes as indicated by a decrease in the number of sequence reads (Figure 2A). Moreover, we found that the overall composition of gut microbes is similar between sleep deprived flies and their non-deprived controls. Although none of the comparisons of the relative abundances between the different bacterial strains yielded a significant p-value after multiple comparison corrections, 9 bacterial strains exhibited interesting trends. A closer look at the change in relative abundances for each of these strains with respect to non-sleep deprived flies indicated that a bacterium from the Alteromonadaceae family was increased by sleep deprivation (Figure 2B). Moreover, 2 strains, one from the Neisseriaceae family and another from the Stenotrophomonas genus
exhibited a decrease in response to sleep deprivation (Figure 2B). These results suggest that sleep is associated with gut microbial changes regardless of circadian time and light-dark cycle. However, whether these changes play a role in sleep regulation is unknown.

3.3 A global reduction in gut microbes and increased bacteria from the *Alteromonadaceae* family are linked to sleep homeostasis

We hypothesized that if the observed changes in the number and composition of gut microbes play a role in sleep regulation, then mutants with defects in sleep regulation should have defects in sleep-deprivation induced gut microbial changes. To test this hypothesis, we examined a transgenic fly (Pumilio RNAi flies) with altered sleep homeostasis using the same protocol described above and determined gut microbial changes in response to sleep deprivation. The specific phenotype of Pumilio RNAi flies is that they lack the sleep rebound or compensatory sleep normally seen after sleep deprivation (De Jesus-Olmos et al., 2015). We predicted that gut microbial changes specifically involved in sleep compensation will be absent or dramatically reduced in Pumilio RNAi flies.

Consistent with our hypothesis, we found that Pumilio RNAi flies do not show the change in the number of gut microbes observed in control flies (compare Figure 2A and 3A). Moreover, the increase in relative abundance of bacteria from the *Alteromonadaceae* family was also absent in Pumilio RNAi flies (compare Figure 2B and 3B). In contrast, the decreased in bacteria from the *Pseudomonas* genus was observed in both *Drosophila* strains suggesting that this change is either not related to sleep, or is associated with an aspect of sleep regulation that is not altered in the Pumilio RNAi knockdown. Interestingly, there were changes in 6 bacterial groups in the mutant
flies that were not observed in normal sleep deprived flies: from the genus *Aquimarina*, the genus *Tenacibaculum*, genus *Verrucomicrobiun*, the *Alcaligenaceae* family and the *Koribacteraceae* family. Taken together these results suggest that the global reduction in gut microbes and the increase in bacteria from the *Alteromonadaceae* family may play an important role in sleep homeostasis.
Figure 1: Specific bacteria change as a function of time of day.

(A) The total number of sequences reads from the night (n=10) and day (n=10) group shows an increase in sequence during the day (P value = 0.0035). The night samples were obtained at ZT-21 and the day samples were obtained at ZT-5. (B) Taxonomy summary bar charts showing relative abundance of night and day gut microbiota.
composition with bacteria class group labeled. (C) Percent change of relative abundance of specific bacteria OTU. Analysis of specific bacteria obtained from QIIME OTU significance analysis. Significant changes were observed in two bacteria groups: (1) with an increase of *Saprospiraceae* family in the deprived flies and a decrease in the (7) *Pseudomonas* genus in the deprived flies. The significant bacteria are indicated in figure B.
Figure 2: Specific bacteria change as a function of sleep

(A) The total number of sequences reads from the Non-deprived (n=23) vs. Deprived (n=25) flies group shows an increase in sequence reads in flies that have been deprived (P value = 0.0390). (B) Percent change of relative abundance of specific bacteria OTU. Analysis of specific bacteria obtained from QIIME OTU significant analysis. Significant changes were observed in three bacteria groups: There was an increase in the Alteromonadaceae family (P value = 0.0164), while we saw a decreases in the Neisseriaceae family (P value = 0.0193) and the Stenotrophomonas genus (P value = 0.0110).

Figure 3: Changes in bacteria observed after sleep deprivation are abolished in mutant flies with impaired sleep compensation

(A) The total number of sequences reads from the Non-deprived (n=29) vs. Deprived (n=43) flies with Pumilio knockdown show no change. (B) Percent change of relative abundance of specific
bacteria OTU. Analysis of specific bacteria obtained from QIIME OTU significant analysis. Significant changes were observed in six bacteria groups: There was an increase in the *Aquimarina* genus (P value = 0.0460), the *Tenacibaculim* genus (P value = 0.0045), the *Alcaligenaceae* family (P value = 0.0085) and the *Verrucomicrobiium* genus (P value = 0.0434), while there was a decrease in two bacteria, *Koribacteraceae* family (P value = 0.0079) and the DA101 genus (P value = 0.0065).

4. Discussion

Despite mayor advantages in our knowledge of the neural circuits, genes and biological processes involved in sleep regulation, the homeostatic control of sleep behavior remains poorly understood. Early classical experiments transferring cerebrospinal fluid (CSF) from sleep-deprived animals into rested non-deprived animals established that the homeostatic control of sleep involved the accumulation of sleep-inducing substances (reviewed in Obal and Krueger 2003). Later studies identified fragments of bacterial cell wall products called muramyl peptides (MPs) as important sleep-inducing substances (Krueger et al., 1982). Although it was known that MPs could be produced by phagocytosis of gram-negative and gram-positive bacteria, whether the bacterial source came from opportunistic infections, endogenous bacteria or contamination has been the subject of a long-standing debate and speculation. Here we take advantage of the sequencing tools available in our genomic era to shine light into this issue. We show for the first time that: 1) the amount and composition of gut microbes changes between sleep and wake flies; 2) sleep deprivation leads to a global decrease in the number of gut microbes and alters the relative abundance of specific bacterial strains and; 3) sleep homeostasis is linked to the global reduction in gut microbes and an increase in bacteria from the *Alteromonadaceae* family. Taken together,
these results indicate that gut microbes play a role in both normal sleep regulation and the response to sleep deprivation.

By integrating our findings with other results in the sleep literature, the following model of sleep regulation emerges: 1) the circadian network regulates the activity of the innate immune system such that macrophages regulating commensal bacterial communities in the gut are more active during the night (Stone et al., 2012); 2) Phagocytosis of gut microbes results in the release of muramyl peptides and cytokines into the hemolymph (insect blood) that induce sleep at night; 3) In the morning, the circadian activation of macrophages is reduced, muramyl peptide and cytokine levels drop and the animal wakes up. Although this model makes sense and nicely explains our observations, it is important to know that there are several extrapolations and speculations. In step 1 and 2, it is extrapolated from the mammalian literature that macrophages regulate the levels of gut microbes (Mowat and Bain., 2011; Harrison and Maloy., 2011) and muramyl peptides are also produced by Drosophila macrophages and that they induce sleep. Although there are no studies showing muramyl peptides in Drosophila, muramyl peptide transporters have been found in their macrophages (Charrière et al., 2010).

In terms of the response to sleep deprivation, we propose that sleep deprivation activates, rather than suppress, the innate immune system and leads to increased gut microbe-phagocytosis, and in the same fashion as described above, sleep is induced. Although the idea that sleep deprivation activates immunity instead of suppressing it sounds counterintuitive, there is evidence from flies, rodents and humans supporting this finding. For example in flies, it has been shown that the bacterial load of non-deprived flies that have been experimentally infected with bacteria is much higher than sleep-deprived flies.
(Williams et al., 2007). Moreover, in both humans and rodents chronic sleep deprivation has been associated with increase circulating levels of pro-inflammatory cytokines which is indicative of immune activation rather than suppression (Mullington et al., 2010; Imeri and Opp., 2009). However, other studies indicate that T cells function and proliferation are reduced in response to sleep deprivation (Bollinger et al., 2009). A possible explanation for these apparent contradictions is that innate immunity is enhanced by sleep deprivation while adaptive immunity is suppressed.

Taken together, our results indicate that gut microbes play a role in both normal sleep regulation and the response to sleep deprivation. Although we did not measure immunity, that fact that our findings nicely matches with previously described immunity changes, suggest that the immune system and specifically macrophages play a major role in this regulation. Given the alarming increases in the prevalence of chronic sleep deprivation and related disorders in our society and the accessibility of gut microbes, our findings could provide the basis for both diagnostic tools for sleep conditions as well as novel treatments.
Chapter III

The Effect of Antibiotic Treatment on *Drosophila melanogaster*’s Sleep Patterns is Dependent on the Time of Antibiotic Exposure
Abstract

Although indigenous microbes regulate the development and function of biological systems that are known to regulate sleep, whether microbes alter sleep behavior remains unknown. Here we use the fruit fly, *Drosophila melanogaster*, as a model system to understand the interaction between sleep behavior and indigenous microbes. Our findings show that antibiotics treatment during development (embryonic and larval stages) decrease sleep behavior while the same treatment during the adult stage increase sleep behavior. Analyses of the sleep structure revealed that developmental and adult antibiotic treatment have opposite effects on sleep episode duration, locomotor activity and on sleep latency. Sleep episode duration is decreased and sleep latency is unaffected by developmental antibiotic exposure. In contrast, adult antibiotic exposure leads to increased sleep episode duration with a decrease in sleep latency and locomotor activity. Interestingly, antibiotic treatment seems to increase levels of synaptic proteins regardless of the developmental stage. Our behavioral results suggest that developmental and adult exposure to antibiotics alter sleep behavior via different mechanisms. Taken together, our findings indicate that intestinal microbes play a role in the functioning and development of the neural circuits involved in sleep regulation.
1. Introduction

Sleep behavior has been observed in a wide range of animals. Considering the vulnerability that results from this state, it could indicate that important functions for survival are occurring during these hours. Particular importance has been associated with brain function. It has been found, for example, that during sleep the interstitial space found in the brain increases by 60% improving the flow of cerebrospinal fluid (CSF) in the area and the removal of accumulated brain metabolite waste (Xie et al., 2013). The consequences of sleep is also associated with brain function deficiency such as learning and memory impairment (Havekes, Vecsey, & Abel, 2012; McCoy & Strecker, 2011; Prince et al., 2013). The adverse effects of sleep lost are also observed in other areas of the body such as in the metabolism (Davies et al., 2014; Van Cauter, Spiegel, Tasali, & Leproult, 2008) and the immune system (Bollinger et al., 2009; M. R. Irwin et al., 2008; Mullington, Haack, Toth, Serrador, & Meier-Ewert, 2009).

Sleep homeostasis is affected by circadian oscillation of hormones, neurotransmitters and other sleep promoting factors in various pathways (Cirelli, 2009; Obal Jr. & Krueger, 2003). The immune system produces some sleep promoting substances such as Interlukin-1 (II-1) and tumor necrosis factor (TNF), (Kapsimalis et al., 2008; James M Krueger et al., 2007; James M Krueger, 2008). The effect of the immune system on sleep induction is seen, for example, when an infection provokes an immune response that leads to the subsequent release of pro-inflammatory cytokines (Kuo et al., 2010; Opp, 2005). Another sleep inducing substance was discovered in early studies using sleep-deprived animals. It was found that when cerebrospinal fluids (CFS) from sleep-deprived goats was injected to rested rats, it increased sleep in the rats.
Subsequent studies described this sleep inducing substance as Factor S (J M Krueger et al., 1978), which was later found in human urine and identified as muramyl peptides (MP) (J M Krueger et al., 1982; J. M. Krueger et al., 1980). MPs are components of the peptidoglycan cell walls of bacteria that can be found as a result of bacterial metabolism or by the digestion of bacteria by phagocytic cells, such as macrophages (Johannsen et al., 1991; Pabst et al., 1999). In addition MPs can also cause an immune response by activation of macrophages leading to the release of cytokines including IL1 (Dinarello & Krueger, 1986). Although we would expect to find MPs as a result of the immune response to an infection, the presence of MPs in healthy sleep deprived animals is unknown.

A possible source for these MP could be from the commensal bacteria found in the gut. It has been calculated that the bacterial cells in this area alone surpasses the number of eukaryotic cells that compose the body by ten times and have been (Gill et al., 2006). Accumulating evidence indicates that the indigenous microbiota regulates a wide range of behaviors. This regulation seems to involve changes in neurotransmitters systems and growth factors as well as endocrine and immune system alterations (Bercik et al., 2011; Clarke et al., 2010; De Araujo, Ferreira, Tellez, Ren, & Yeckel, 2012; Diaz Heijtz et al., 2011; Ivanov & Honda, 2012; K. M. Neufeld et al., 2011; Purchiaroni et al., 2013; Round & Mazmanian, 2009; Tlaskalova-Hogenova et al., 2005). For example, it has been shown that germ-free or antibiotic treated animals exhibit altered anxiety and depressive-like behaviors, stress responses, mating preferences and social recognition (Bercik et al., 2011; Bravo et al., 2011; Diaz Heijtz et al., 2011; Dillon, Vennard, & Charnley, 2000; Lyte, 2013; K. M. Neufeld, Kang, Bienenstock, & Foster, 2011; Theis,
Schmidt, & Holekamp, 2012). These behavioral changes are accompanied by alterations in hormones and neuromodulators such as corticosterone, BDNF, dopamine, GABA, serotonin and noradrenaline (Bercik et al., 2010; Bravo et al., 2011; Diaz Heijtz et al., 2011; Lyte, 2013; K. M. Neufeld et al., 2011). Certain members of the gut microbe composition have been found to produce metabolic substances that are known to influence sleep regulation such as the neurotransmitter GABA, serotonin and its precursor, tryptophan, dopamine and acetylcholine (Barrett, Ross, O’Toole, Fitzgerald, & Stanton, 2012; Cryan & Dinan, 2012; L. Desbonnet et al., 2010; Stephenson & Rowatt, 1947).

Although indigenous microbes regulate a number of biological processes such as circadian rhythm, the immune system and various neurotransmitter pathways that are known to regulate sleep, whether microbes alter sleep behavior remains unknown (Barrett et al., 2012; Cryan & Dinan, 2012; L. Desbonnet et al., 2010; Stephenson & Rowatt, 1947). Regarding circadian rhythms, it has been shown feeding cycles leads to oscillation of gut microbial communities and that this oscillation serve as a time giver to the intestinal epithelial cells (IEC)(Asher & Sassone-Corsi, 2015; Mukherji et al., 2013). Importantly, it was shown that IEC’s clock impairment disrupts the levels and cycling of corticosterone and metabolic homeostasis at a systemic level (Mukherji 2013, Thaiss 2014).

The effects that the gut microbiota could be having on the development of sleep related neuronal circuits or sleep behavior, have not been studied to date. Based on what is known about the gut microbiota’s ability to influence behavior, central nervous system protein expressions and the production of metabolites that are known to be involved in
sleep homeostasis; we hypothesized that this community of commensal microbes could be influencing normal sleep regulation. To test this hypothesis we studied the sleep patterns of the fruit fly, *Drosophila melanogaster*, after treatment with an antibiotic cocktail during the developmental stage and as adults. The fruit file is a well understood model to study sleep patterns and structures comparable to mammals (Cirelli, 2009; Shaw et al., 2000). Preliminarily, we also looked at the levels of expression of the synaptic protein DLG, which has been linked to sleep behavior (Gilestro, Tononi, & Cirelli, 2009).
2. Material and Methods

2.1 Antibiotic treatment

The experiment was performed using the wild type strain of *Drosophila melanogaster* Oregon R. Flies were raised at 25°C on banana-based medium (Heed’s banana medium). Treatment with antibiotics was performed by adding a cocktail of wide spectrum antibiotics in the following concentrations: 50 µg/mL of tetracycline, 200 µg/mL of rifampicin and 100 µg/mL of streptomycin (Sharon et al., 2010). These antibiotics were added to the food once it had cooled down before solidification. Twenty couples were placed per bottle and left for four days to deposit eggs. For a second generation of treatment, twenty couples of the flies reared in antibiotic treatment were transferred to a fresh bottle of food containing the same antibiotic cocktail as before and left to deposit eggs for four days. The control group was flies reared in conventional banana base food without antibiotics.

2.2 Experimental Groups

The following experimental groups were prepared. Experimental groups were composed of adult females. For this experiment females were chosen over males due to the difference in sleep patterns between males and females.

Control Group- Flies were reared in conventional banana medium (Heed’s banana medium) without antibiotic. Four to five days old adult flies were transferred to 5% sucrose 2% agar food source in tubes for sleep assays (Figure 1A) (Agosto et al., 2008). Developmental group- Flies were reared in the antibiotic medium for two generations. Second generation adult flies (4-5 days old) were transferred to 5% sucrose plus 2% agar food source in tubes for sleep assays (Figure 1B). Adult group- Flies were reared in conventional banana medium
without antibiotic. Four to five days old adult flies were transferred to 5% sucrose plus 2% agar food source with antibiotic cocktail in tubes for sleep assays (Figure 1C). Development + Adult Group- Flies were reared in the antibiotic medium for two generations. Second generation adult flies (4-5 days old) were transferred to 5% sucrose plus 2% agar food source with antibiotics in tubes for sleep assay (Figure 1D).

2.3 Sleep Assays

To monitor sleep behavior the Drosophila Monitoring System (DAMS) form Trikinetics was used as previously described (Chiu et al., 2010; Shaw et al., 2000). The experimental and the control groups were placed in a programmable incubator (Percival Model I-30BLL) set at a 12:12 light-dark cycle with a temperature of 25°C and at 80% humidity. After placing the flies in the monitors and incubator, they were allowed to adapt for two days. Sleep behavior was recorded for the following four days and this data was averaged and analyzed using the MATLAB software and PRISM statistical analysis software.

2.4 Western Blot Analysis of Synaptic Protein DLG

Previous studies have found a correlation between certain synaptic proteins such as the postsynaptic protein DLG and sleep (Gilestro et al., 2009). This group found that upon sleep deprivation, DLG levels increase while levels decrease after sleep. To see if DLG levels are affected with antibiotic treatment, levels of expression were measured. Fly heads were collected in groups of 5 in a sterile microtube keeping samples on ice. The homogenizing buffer solution used was prepared using the formula form Zang et al. 2010 as follows: 100 mM KCl, 20 mM HEPES, 5% Glycerol, 10mM EDTA, 0.1% Triton, 1mM DTT and 1µL of Protease Cocktail per every 5µL of buffer solution before
use. 60 µL of buffer were added to the microtube with the fly heads. The heads were homogenized while keeping it on ice. The tube was left for one minute on ice then 40 µL more of buffer was added for a total of 100 µL of solution and homogenized again. The tubes were centrifuged at 500 rcf for 5 minutes. The supernatant were transferred to a fresh microtube and stored at -70ºC until use.

Protein determination assay was carried out using DC Protein Assay Kit from BioRad following the kits protocol. Once protein concentrations were determined, a concentration of 5 µg of protein was loaded on 10% polyacrylamide gel at 150V for 40 min. The proteins were transferred to nitrocellulose using the Turbo Trans Blot. The primary antibody used was mouse anti-DLG (1:2500) with the secondary antibody IRDye 800CW goat anti-mouse from LI-COR. The results were observed using the Lanco Odyssey System and the intensity of the bands were quantified using LI-COR imaging program.
3. Results

3.1 Baseline Sleep in Flies Exposed to Antibiotic during Development

To determine how antibiotic exposure during the developmental stage affected sleep behavior in *Drosophila*, the sleep pattern of flies reared in antibiotic supplemented food source was analyzed. These flies were compared to flies reared on control food that were transferred to sucrose/agar food, using the DAMS. Sleep behavior was measured for four days after a two-day adjustment period. In figure 2A, the sleep plot indicates a significant change in sleep behavior during the night as well as in the day. Overall the antibiotic treated flies tend to sleep significantly less during the night and day (figure 2B). We aimed to understand the sleep factors that can be accounting for the decrease observed in the antibiotic treated flies. When analyzing the sleep structure we observe that antibiotic treated flies do not have changes in the sleep onset (figure 3A) but have shorter sleep episodes that can account for the decrease of sleep (figure 3B). Through the night these shorter sleep episodes are more frequent (figure 3C) indicating that the sleep that is obtained is very fragmented. It is also observed that the treated flies are more active when awake (figure 3D).

3.2 Baseline Sleep in Flies Treated with Antibiotics During the Adult Stage

To determine how antibiotic exposure of a developed adult fly affects sleep, flies reared in conventional banana base food source were transferred to 5% sucrose plus 2% agar with the antibiotic cocktail, for sleep analysis in the DAMS. These flies were compared to control flies with no exposure to antibiotics. Sleep behavior was measured after two days of adjustment. Data was analyzed from four consecutive days. As seen in figure 4A, the sleep plot shows a trend for treated flies to sleep more during the day.
while we see no significant change in sleep behavior during the night. The average of
total sleep significantly increased for antibiotic treated flies during the day while no
changes in total sleep during the night were observed (figure 4B). These changes in
daytime sleep are due to shorter time for sleep onset of the antibiotic treated flies (figure
5A) as well as longer sleep episodes during the day. We found no changes in the night’s
sleep episodes (figure 5B). The increase in total sleep observed is not due to more sleep
episodes, as they are similar between the antibiotic treated flies vs. the control (figure
5C). Aside from sleeping more during the day, the treated flies also have a decrease in
activity in the periods of arousal (figure 5D).

3.3 Baseline Sleep in Flies Treated with Antibiotics during the Development and
Maintained in Antibiotics during the Adult Stage

In this experimental group, the sleep patterns of flies that were reared in
antibiotic supplemented medium were transferred to 5% sucrose plus 2% agar with the
antibiotic mixture was analyzed. After two days of adjustment period, data was analyzed
for the next four days. In the sleep plot, a change in sleep behavior can be seen in the
antibiotic treated flies (figure 6A) with a decrease in total sleep during the night as well
as in the day (figure 6B). Interestingly, treated flies have a faster sleep onset after lights
on during the day, although, change in latency was not seen after lights off in the night
(figure 7A). However, it can be seen that the decrease in total sleep is due to shorter
sleep episodes during the night and the day (figure 7B). There is a tendency for an
increase in the number of sleep episodes, indicating some fragmentation of sleep,
although the change is not significant (figure 7C). The moments of wakefulness are
more active for the flies treated (figure 7D).
3.4 Expression of synaptic protein DLG in flies treated with antibiotics

Seeing the changes in sleep patterns of flies treated with antibiotic, we looked at the levels of the synaptic protein DLG. In previous studies, levels of synaptic proteins would rise during the day and decrease after sleep. In sleep deprivation, the levels of these proteins increase (Gilestro et al., 2009). Protein was extracted from a group of 4 heads per sample to perform western blots. The bands were visualized using and fluorophore antibody IRDye 800CW from LI-COR and the Odyssey Imaging System (figure 8A). The quantification of the bands was graphed using Prism (figure 8B). In our preliminary analysis the levels of DLG of flies treated with antibiotic tend to increase significantly independent of when the antibiotic exposure took place. Although total sleep in flies treated during development tends to decrease and flies exposed as adults tends to increase, we would expect to find an increase in expression in the development group and a decrease in the adult group. These findings could indicate that the mechanisms by which the microbiota is influencing sleep could be different.
Figure 1: Experimental Design for Antibiotic Treatment during development and adult stage.

Antibiotic treatment of flies consisted of 50 µg/mL of tetracycline, 200 µg/mL of rifampicin and 100 µg/mL of streptomycin. (A) The control group that did not receive any antibiotics. (B) The development group received antibiotic exposure in the food during the larvae stage until they emerged from the pupa; adults were removed from antibiotic exposure. (C) For the adult group larvae were reared in non-antibiotic conventional food medium; then adults were exposed to antibiotics. (D) For the Development + Adult group larvae were reared in antibiotics and the adults maintained in antibiotic exposure.
Figure 2: Antibiotic treatment during development lead to decreased sleep behavior in adult flies.

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (gray square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day. Sleep is shown as the average of sleep per 30 minutes of flies that have been treated with an antibiotic cocktail during developmental stages vs. control. Treated flies sleep less than flies that have not been exposed to antibiotics during a 24-hour day (mean ± SEM, n=47 control flies and 51 antibiotic treated flies). Two-way repeated-measure ANOVA identified a significant change in time x antibiotic treatment ($F_{[47,4512]} = 8.246, p < 0.0001$).

(B) Effect of antibiotic exposure on total sleep during the night and day. White bars indicate control flies and gray bars indicate antibiotic treated flies. Total sleep is represented as the average of sleep in 12 hours. Flies exposed to the antibiotics show to have less total sleep during the night as well as during the day. Asterisk (*) indicates statistical significance. Repeated-measure ANOVA showed a significant change in the night (p=0.0064), day (p < 0.0001) as well as interaction ($F_{[1.96]} = 5.872, p=0.0173$).
Figure 3: Antibiotics during development lead to decreased sleep duration and hyperactivity.
(A) Sleep latency after lights off (night) and after lights on (day) indicates the average of minutes for control flies (white bars) and treated flies (gray bars) to demonstrate sleep onset. No significant changes were observed when comparing control flies to those treated with the antibiotics. (B) Max sleep episode duration of flies during the night and the day. Flies treated (gray bars) showed a reduction in the duration of sleep bouts during the night (p = 0.0024) as well as in the day (p < 0.0001). Asterisk (*) indicates statistical significance. (C) Number of sleep episodes during the night and the day. Antibiotic treated flies showed a significant increase in number of sleep episodes (p = 0.0317) when compared to non-treated flies. No changes were observed in the number of sleep episodes in the day. (D) Mean locomotive activity of flies during the waking period during the night and day. The treated flies showed a significant increase in activity during the night (p < 0.0001) and during the day (p < 0.0001) with significant interaction between night and day (F[1,96]=11.75, p = 0.0009).
Figure 4: Antibiotic treatment during the adult stage, selectively increase daytime sleep behavior.

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (gray square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day. Sleep is shown as the average of sleep per 30 minutes of flies that have been treated with an antibiotic cocktail during adult stages vs. control. Adult flies that have been treated with an antibiotic cocktail sleep more than flies that have not been exposed to antibiotics during a 24 hour day (mean ± SEM, n=47 flies in control and 43 flies in antibiotic treated group). Two-way repeated-measure ANOVA identified a significant change in time x antibiotic treatment ($F_{[47,4136]} = 3.432, p < 0.0001$). (B) Effect of antibiotic exposure on total sleep during the night and day. White bars indicate control flies and gray bars indicate antibiotic treated flies. Total sleep is represented as the average of sleep in 12 hours. Repeated measure ANOVA shows that the adult flies exposed to the antibiotics have a total sleep significantly higher during the day ($p < 0.0001$) with no significant difference in total sleep during the night. There is a significant interaction between night and day ($F_{[1,88]} = 9.076, p= 0.0034$). Asterisk (*) indicates significance.
Figure 5: Antibiotics during the adult stage lead to decreased sleep latency and increase in sleep duration.

(A) Sleep latency after lights off (night) and after lights on (day) indicates the average of minutes for sleep onset in control flies (white bars) and treated flies (gray bars). Treated flies have a decrease sleep latency in the day (p<0.0001) with no change observed during the night. There is significant interaction observed between night and day (F_{[1.88]}=29.81, p<0.0001). Asterisk (*) indicates significance.

(B) Max sleep episode duration of flies during the night and the day. Exposed flies showed longer sleep episodes during the day (p = 0.0003) with no changes in the night’s episode duration. A significant interaction was observed from night to day (F_{[1.88]}= 4.065, p=0.0468).

(C) Number of sleep episodes during the night and the day show no significant changes.

(D) Mean locomotive activity of flies during the waking period during the night and day show only a significant decrease during the day (p=0.0224).
Figure 6: Combining development and adult stage antibiotic treatment leads to decreased sleep behavior.

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (black square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day. Sleep is shown as the average of sleep per 30 minutes of flies that have been treated with an antibiotic cocktail during development through adult stage vs. control. Treated flies show to sleep less then flies that have not been exposed to antibiotics during a 24 hour day (mean ± SEM, n=47 flies in control and 50 flies in antibiotic treated group). Two-way repeated-measure ANOVA identified a significant change in time x antibiotic treatment ($F_{[47,4465]} = 9.340$, $p < 0.0001$). (B) Effect of antibiotic exposure on total sleep during the night and day. White bars indicate control flies and black bars indicate antibiotic treated flies. Total sleep is represented as the average of sleep in 12 hours. Flies treated with antibiotics sleep less during the night ($p=0.0334$) as well as during the day ($p<0.0001$) with an of interaction $F_{[1,95]}= 4.305$, $p= 0.0407$. Asterisk (*) indicates significance.
Figure 7: Sleep structure analysis reveals latency effects similar to adult treatment while sleep duration and activity effects resemble developmental antibiotic exposure.

(A) Sleep latency after lights off (night) and after lights on (day) indicates the average of minutes for sleep on set in control flies (white bars) and treated flies (black bars). Treated flies have lower sleep latency in the day (p<0.0001) with no significant change observed during the night. Interaction is observed between night and day ($F_{[1.95]} = 5.358$, p=0.0228). Asterisk (*) indicates significance. 

(B) Max sleep episode duration of flies during the night and the day. Treated flies show to have shorter sleep episodes during the night (p=0.0307) and during the day (p<0.0001). A significant interaction was observed from night to day ($F_{[1.95]} = 4.437$, p=0.0378). 

(C) No significant changes were observed in total number of sleep episodes neither in night nor day. 

(D) Mean locomotive activity of flies during the waking period during the night and day. Flies that were treated showed to have higher activating during the waking periods in the night (p<0.0001) as well as in the day (p=0.0094). Significant interaction between night and day was also observed ($F_{[1.95]} = 8.496$, p=0.0044).
Figure 8: In preliminary results, levels of synaptic protein tend to increase with antibiotic exposure.

(A) Western blot for detection of the synaptic protein DLG from Drosophila head lysate (5 heads per sample). Protein was detected with mouse anti-DLG, followed by IRDye 800 goat anti-mouse for control flies with no antibiotic exposure (Ctrl), for flies that received antibiotic exposure only during developmental stages (Dev), flies exposed as adults only (Adult) and flies that were maintained in the treatment all through development and as adults (Dev+Adult). B) Quantification of fluorescence intensity from western blot (A) (n=2). Analysis of one-way ANOVA showed no significance due to n value although, a tendency of an increase in DLG is seen in flies exposed to antibiotics.
4. Discussion

Antibiotic treatment has been found to alter gut microbiota (Dethlefsen, Huse, Sogin, & Relman, 2008; Jernberg, Löfmark, Edlund, & Jansson, 2007; Willing, Russell, & Finlay, 2011). The absence of microbiota colonization soon after birth correlates with deficiencies in the developmental aspects of the intestine and immunity such as the development of intestinal goblet cells which produce the mucus layer (Bengmark, 2013; Deplancke & Gaskins, 2001; Kelly et al., 2007; Russell et al., 2012; Szentkuti, Riedesel, Enss, Gaertner, & Von Engelhardt, 1990). Interruption of the flora by antibiotics also cause dysbiosis which have been linked to a series of disorders such as depression, irritable bowl syndrome, and autism (de Theije et al., 2013; Kennedy et al., 2014; Naseribafrouei et al., 2014). These types of disorders have also been linked to sleep disruptions but a link between the gut microbes and sleep has not been investigated (Ali, Choe, Awab, Wagener, & Orr, 2013; Mayes et al., 2009; Nutt, Wilson, & Paterson, 2008).

In this study, we show that altering or eliminating gut microbiota from Drosophila melanogaster by treating them with an antibiotic cocktail can also have an effect on sleep behavior. Our results show that sleep behavior of Drosophila treated with antibiotics has an opposite effect on total sleep depending on the life stage of the exposure. Figure 2A and figure 5A show that flies that received treatment during development tend to sleep less during the night as well as the day while those that received treatment only as adult (figure 4A) tend to sleep more, with a significant increase in sleep during the day. Although the changes in microbiota were not measured after antibiotic treatment, the difference in sleep behavior from adults to development
could be due to a different type of shift in the microbiota population as seen by Russell et al. (2012) after treatment of antibiotics. Our findings show that the moment at which the intestinal microbiota is manipulated will cause a different effect on the host. It has been seen in mice that treatment during the neonatal stage greatly decrease diversity in the gut bacterial composition (Russell et al., 2012). A possible mechanism by which these changes could be influencing sleep could be due to bacterial products of the gastrointestinal track which in turn are known to be part of sleep regulation pathways (Barrett et al., 2012; Cryan & Dinan, 2012; L. Desbonnet et al., 2010; Stephenson & Rowatt, 1947). One of these bacterial products is GABA, an important inhibitory neurotransmitter and key player in promoting sleep, also important in Drosophila sleep regulation (Agosto et al., 2008; Gottesmann, 2002). A bacteria species commonly found in Drosophila is Lactobacillus brevis which has been found to produce GABA (Barrett et al., 2012; Wong, Ng, & Douglas, 2011). With lower concentrations of this neurotransmitter we would expect to have a weaker drive for sleep behavior. The preoptic area (POA) is a GABAergic region in the hypothalamus that is active during sleep. In rats, lesions of the POA showed similar changes in sleep to the ones observed in the flies that received antibiotic treatment during development (John & Kumar, 1998). In flies that have been treated with antibiotic during development we observed a decrease in total sleep during the night as well as during the day (figure 2). This decrease in total sleep is due to shorter sleep episodes during the night and day with fragmentation of the night episodes as well as an increase in activity in the waking moments (figure 3). In the John and Kumar (1998) study, the lesions of the POA provoked a decrease in total sleep with shorter sleep episodes while an increase in
locomotive activity was observed. This could suggest that the changes in microbiota during early stages in the life of the fly are affecting the normal development of sleep regulation neurons.

MPs, fragments of bacterial cell wall, have associated with sleep MP. Another possible pathway by which the gut microbes could be influencing sleep is by way of the MPs from the bacterial cell walls (Johannsen et al., 1991; J M Krueger et al., 1982; J. M. Krueger et al., 1980; Pabst et al., 1999). Macrophages have been found to liberate MPs after the process of phagocytosis (Johannsen et al., 1991). The correlation between elimination of the microbiota by treatment with antibiotics during development and the decrease in total sleep (figure 2) could indicate that the presence of MPs could be playing a role in sleep behavior. Taking a closer look at the sleep structure we see that the duration of the sleep episodes are shorter (figure 3B) which would suggest that MPs are influencing the continuance of sleep once sleep onset commences. Research focused on how MPs interacts with the CNS found that these peptides can affect synaptic receptors associated with sleep regulation such as provoking an increase in GABA-induced currents which would promote sleep (Grigoriev et al., 2008; Saper, Scammell, & Lu, 2005). Although it has been shown that MPs are able to interact in the CNS receptors, it has not been considered if the MPs are originating from the intestinal microbes.

However, an increase in MPs could be expected as a result of a treatment with antibiotics. Such an increase in MPs would provoke an increase in sleep not only by its effects on related neuronal circuit (Grigoriev et al., 2008; Saper et al., 2005) but also by activating an immune response that results in the release of sleep promoting
cytokines (Dinarello & Krueger, 1986; Pabst et al., 1999; Silverman et al., 1985). The results observed in flies treated as adults show this expected sleep increase. We observed that antibiotic treatment of adults lead to an increase in total sleep during the day (figure 4) as a result of faster sleep onset (figure 5A) and longer sleep episodes (figure 5B). Similar sleep behavior is observed in flies that are suffering from an infection that activate an immune response (Kuo et al., 2010). In a study using Drosophila, it was seen that infection or injury would promote an increase in sleep behavior in the day (Kuo et al., 2010).

The disruption of the gut microbiota composition by the treatment of antibiotic can cause dysbiosis which can lead to health complications (Hawrelak and Myers 2004; Jernberg et al. 2007; Willing, Russell, and Finlay 2011). The problem that could result from this dysbiosis could be observe as the overgrowth of the resistant bacterial strains, the build-up of toxic metabolites and altered immune response by interruption of gut homeostasis (Willing et al., 2011). A possible consequence of this problems could lead to inflammation and gut permeability that could facilitate the transfer of gut microbes to the rest of the body provoking an immune response and as a consequence an increase in sleep (Jiang et al., 2015; Kuo et al., 2010; Mukherji et al., 2013; Rescigno, 2011).

The group of flies that was reared in antibiotics and kept in the treatment as adults showed similar sleep patterns as those that were only exposed to antibiotics during development. They showed and decrease in total sleep due to shorter sleep episodes as well as an increase in activity during the waking periods (figures 2,3,6 and 7). While the development group does not show a change in sleep onset (figure 3A), the ones kept on antibiotics show faster sleep onset during the day (figure 7A), similar to
results seen in those flies exposed to antibiotics as adults (figure 5A). The decrease in sleep latency is seen in flies that are in antibiotic supplemented food at the time of sleep analysis, this could suggest that the antibiotics per se are promoting faster sleep onset during the day. Further studies are needed to elucidate if this is the case.

It has been observed in Drosophila that sleep lost can increase synaptic protein markers, such as Discs-large (DLG) and Bruchpilot (Gilestro et al., 2009). Studies with germ free mice also show that there is an increase in the synaptic proteins, Synaptophysin and PSD-95, in the striatum (Diaz Heijtz et al., 2011). As a preliminary study we looked at DLG expression of the treated flies. These first results indicate that there in an increase of DLG in all experimental fly groups independent of when they were treated with the antibiotics (figure 8). Although these are just preliminary results, this could indicate that synaptic proteins may indeed be influencing synaptic proteins expression and that the mechanism by which sleep is affected in adult treated flies (figure 4), that sleep more, is different form flies that are treated during development, that sleep less (figure 2 and 6). Further experimental data must be collected to study this further.

To the best of our knowledge, the effect that the microbiota could have on sleep has not been studied. The results presented here indicate that the intestinal bacteria may play a role in sleep regulation and structure. The microbiota could be providing molecular products that influence sleep behavior and/or it could influence early development of sleep-wake regions of the CNS that cause a decrease in sleep. Additional work must be done to correlate changes in the CNS after antibiotic treatment during development as well as reintroduction of the microbiota to see if rescue of sleep
behavior is obtained. However, the sleep behavior observed in adult flies treated with antibiotic indicates symptoms similar to an immune response to infection, with an increase in sleep during the day. To confirm dysbiosis in these flies, a study of the composition of the gut microbiota after antibiotic treatment is needed. Also as a future study, sleep behavior of axenic flies needs to be measured to rule out possible effects of the antibiotics.
Chapter IV

Knockdown of the Eater Gene of
Drosophila melanogaster Decrease
Night Total Sleep
Abstract

The immune system is closely linked with sleep behavior. Products of the immune system response such as IL-1 and TNF, have been classified as sleep regulatory substances. Aside from these cytokines being able to promote sleep, immune activity seems to also be regulated by the circadian clock. Here we study *Drosophila melanogaster* as a model to study the role of the immune system on sleep behavior by impairing phagocytosis by knocking down the eater gene by use of RNAi of the eater gene. Our results show that mutant flies that are unable to engulf bacteria have a diminution in sleep during the night due to shorter yet more frequent sleep episodes indicating fragmented night sleep while the day sleep in unaffected.
1. Introduction

In the fruit fly, there are important molecular mechanisms that quickly act to fight off a microbial infection (Kounatidis & Ligoxygakis, 2012). An important component of the immune system are the plasmatocytes, not only for their role in phagocytosis but also for the release of signaling cytokines that activate pathways, such as Toll and JAK/STAT, that result in antimicrobial peptides (AMP) synthesis (Lemaitre, 2004; Rosetto, Engström, Baldari, Telford, & Hultmark, 1995; Stuart & Ezekowtiz, 2008). These cytokines activate the transmembrane receptors Toll and Domeless (for the JAK/STAT pathway) of the fat body, the mammalian equivalent of the liver, which will release the appropriate AMPs into the hemolymph to combat the microbes (Agaisse, Petersen, Boutros, Mathey-Prevot, & Perrimon, 2003; Charroux & Royet, 2010; De Gregorio, Spellman, Tzou, Rubin, & Lemaitre, 2002; Ferrandon, Imler, Hetru, & Hoffmann, 2007; Leulier et al., 2003; Royet, Reichhart, & Hoffmann, 2005). However, before the release of signaling molecules by macrophages, recognition and phagocytosis occurs. The first step in the phagocytosis process is the recognition of the antigen by cell surface receptors (Ferrandon et al., 2007). In Drosophila, plasmatocytes receptors have been identified that are specific for certain antigens. For example PGRP-LC is involved in phagocytosis of gram-negative bacteria (Rämet, Manfruelli, Pearson, Mathey-Prevot, & Ezekowitz, 2002). While other transmembrane proteins, such as Eater, have been associated with the phagocytosis of both gram-positive and gram-negative bacteria (Chung & Kocks, 2011; Kocks et al., 2005). Mutant flies without PGRP-LC are vulnerable to infection with gram-negative but not gram-positive. Mutants without eater were observed to have impaired phagocytic activity with a significant increase in
bacterial load and a lower survival rate after infection (Kocks et al., 2005). The *eater* gene encodes for a protein that is important for the recognition, binding and phagocytosis of bacteria (Chung & Kocks, 2011; Kocks et al., 2005).

The binding of an antigen to receptors activates changes of the actin cytoskeleton and membrane, which surround the bacteria or cell debris forming a vacuole. When it is engulfed in the phagocyte, lysosome fuse to the vacuole releasing enzymes that break down the pathogen or dead cell components. This is now the phagolysosome. The product inside the phagolysosome can be release from the phagocyte by exocytosis (Stuart & Ezekowitz, 2008). Upon activation, the macrophages can secrete cytokines that activate other immune cells for a more effective immune response (Stuart & Ezekowitz, 2008).

Cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF) have been classified as sleep regulatory substances (J M Krueger, Walter, Dinarello, Wolff, & Chedid, 1984; James M Krueger, ObáL, Fang, Kubota, & Taishi, 2001; Takahashi et al., 1999). It is commonly observed that sleep increases with illness. It has been thought that this increase in sleep is a way to help the immune system in the healing process. Studies have indeed identified that an immune response is responsible for the prolongation of sleep behavior (Kuo et al., 2010; Toth & Krueger, 1989). IL-1 and TNF in mice was also shown to play a role as part of regular sleep patterns since inhibiting them show a decrease in sleep behavior (J M Krueger et al., 1998). In the search of the mechanism on how these substances are playing a role in sleep regulation, it has been found that they are able to influence the sleep and wakefulness regulating
system of the central nervous system (CNS) (Brambilla, Franciosi, Opp, & Imeri, 2007; Feleder, Refojo, Nacht, & Moguilevsky, 1998; Imeri et al., 1997; Rada et al., 1991).

The effects of IL-1 are one of the most studied and it has been seen that it is able to have an effect over GABAergic neurons. GABA is an important inhibitory neurotransmitter in the CNS and it’s a principal player in promoting sleep (Gottesmann, 2002). IL-1 is stimulating sleep by increasing the release of GABA which in turn inhibits wake-promoting (excitatory) neurons (Brambilla et al., 2007; Feleder et al., 1998). Studies have also found that this cytokine can decrease the extracellular levels of acetylcholine which is mostly associated with waking activities (Rada et al., 1991). Also, IL-1 has been associated with an increase in levels of adenosine, which has been found to promote sleep perhaps by inhibiting wake-promoting neurons through activation of GABA receptors (Basheer, Strecker, Thakkar, & McCarley, 2004; Mendelson, 2000; Sperlágh, Baranyi, Haskó, & Vizi, 2004).

Although it is well known that components of the immune system are sleep inducing and have been categorized as sleep regulatory substance, there are still many aspects that remain to be elucidated (J M Krueger et al., 1984; James M Krueger et al., 2001; Takahashi et al., 1999). Various studies have confirmed that certain functions of the immune system are linked with the circadian rhythm (Keller et al., 2009; Kuo et al., 2010; Stone et al., 2012). In mutant flies of circadian clock genes show deficient immune response (Keller et al., 2009; Kuo et al., 2010; Stone et al., 2012). Based on the influence that the immune response has on sleep and the how circadian rhythmicity affects the immune response; we hypothesized that phagocytic activity plays a role in sleep behavior regardless of infection. To determine if phagocytic activity plays a role in
sleep regulation when there is no infection present, the gene *eater* was knocked down from *Drosophila* impairing the phagocytic process (Chung & Kocks, 2011; Kocks et al., 2005).
2. Material and Methods

2.1 Fly Stocks

This experiment was performed using two mutant flies obtained from Bloomington Drosophila Stock Center: \( \text{w}^{[118]}; \text{P}\{\text{w}+[\text{+mC}]=\text{Hml-}\text{GAL4.}\text{G}\}\text{6-4 P}\{\text{w}+[\text{+mC}]=\text{UAS-GFP::lacZ.nls}\}\text{15.1P}\{\text{w}+[\text{+mC}]=\text{UAS-GFP.S65T}\}\text{Myo31DF[T2]} \) and Genotype: \( \text{y}[1] \text{ v}[1]; \text{ P}\{\text{y}+[\text{t7.7}]=\text{Trikinetics}\text{TRiP.JF01884}\text{attP2} \). The knockdown was achieved by crossing ten male UAS-\textit{Eater} RNAi flies with ten virgin females Hml-GAL4 (hemocytes marked by GFP) (figure 1). The flies were reared on banana base medium at 25\(^\circ\)C (Heed’s banana medium).

2.2 Sleep Assays

To monitor sleep behavior the \textit{Drosophila} Monitoring System (DAMS) form Trikinetics was used as previously described (Chiu et al., 2010; Shaw et al., 2000). The experimental and the control groups were placed in a programmable incubator (Percival Model I-30BLL) set at a 12:12 light-dark cycle with a temperature of 25\(^\circ\)C and at 80\% humidity. After placing the flies in the monitors and incubator, they were allowed to adapt for two days. Sleep behavior was recorded for the following four days and this data was averaged and analyzed using the MATLAB software and PRISM statistical analysis software.
3. Results

3.1 Baseline Sleep in Flies with Impaired Phagocytosis

To determine if manipulation of macrophage activity alters sleep patterns, an essential gene for macrophage phagocytosis, *Eater*, was knocked down by way of RNA interference in the fly’s hemocytes, the equivalent to macrophages (Figure 1). Eater is an important protein in the eater receptor that’s expressed by phagocytic cells that recognizes gram-positive and gram-negative bacteria sending the signal to engulf the bacteria. The knockdown of eater have decreased phagocytic activity without affecting other types of immune signaling (Kocks et al., 2005).

In this study the sleep behavior of flies with non-functional *Eater* were observed. In the sleep plot we see that there is a decrease in sleep activity during the night (figure 2A). The mutant flies total sleep significantly decrease in the night (figure 2B). When we analyze the sleep structure there was no change in sleep structure (figure 3A). However, the decrease in night sleep is due to shorter more frequent sleep episodes indicating that sleep at night is fractionated, (figure 3B and 3C). There is also significant increase in the flies’ activity during the period they are awake in the night as well as in the day (Figure 3D).
Figure 1: Experimental Procedure for the Generation of Flies with Eater Knockdown

Ten male UAS-Eater RNAi’s were crossed with ten virgin females with an Hml-Gal4 driver. The female Eater knockdown progeny was placed in the behavior monitors to record sleep behavior.
**Figure 2: Eater knockdown flies leads to a decrease in total sleep during the night.**

(A) Standard sleep plot of control (white triangle) and antibiotic treated flies (gray square) in a 12 hour: 12 hour light:dark (LD) periods represented in zeitgeber time (ZT). Gray box represent night and the white area represents day. Sleep is shown as the average of sleep per 30 minutes of flies with eater knockdown vs parental fly with functional phagocytic activity. Flies with impaired phagocytic activity sleep less during the night and seem to show a tendency to sleep slightly less during the day (mean ± SEM, n=121 flies in control and 121 flies in Eater RNAi). Two-way repeated-measure ANOVA identified a significant change in time x Eater RNAi ($F_{[47,11280]} = 16.17, p < 0.0001$). (B) Effect of impaired phagocytic activity on total sleep during the night and day. White bars indicate control flies and black bars indicate Eater RNAi flies. Total sleep is represented as the average of sleep in 12 hours. Repeated-measure ANOVA showed a significant change in total sleep during the night ($p<0.0001$) of flies with eater knockdown. Asterisk (*) indicates statistical significance. There is a significant interaction between night and day ($F_{[1,240]} = 39.09, p<0.0001$).
Figure 3: Eater Knockdown results in shorter more frequent sleep episodes.

(A) Sleep latency after lights off (night) and lights on (day) indicates the average of minutes for sleep onset in control flies (white bars) and Eater RNAi flies (black bars). Eater RNAi flies showed no significant change in the amount of time it took for sleep onset (latency) for the night or day.

(B) Max sleep episode duration of flies during the night and the day. Eater RNAi flies show shorter sleep episodes during the night (p = 0.0142) with no changes in sleep episode duration during the day. A significant interaction was observed from night to day (F[1,240] = 7.201, p=0.0078).

(C) Eater RNAi flies show an increase in sleep episodes during the night (p=0.0138) with no significant change during the day.

(D) Mean locomotive activity of Eater RNAi flies are more active in their waking periods in the night and day (p<0.0001).
4. Discussion

Injury and infection have been shown to increase sleep behavior (Kuo et al., 2010; Toth & Krueger, 1989). This increase in sleep is an effect of the immune response. Cytokines, such as Interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been classified as sleep regulatory substances, among other immune system’s molecules (J M Krueger et al., 1984; James M Krueger et al., 2001; Takahashi et al., 1999). Muramyl peptides (MP), fragment of the bacterial peptidoglycan walls, have also been found to promote sleep (J M Krueger et al., 1982; J. M. Krueger et al., 1980; James M Krueger, 1990). Cytokines are released from activated macrophages among other immune cells and also MPs from the phagocytized bacteria which in turn, are also activators of the immune response (Murray & Stow, 2014; Pabst et al., 1999; Stow, Ching Low, Offenhäuser, & Sangermani, 2009).

With this work we report that impaired phagocytic activity by use of RNA interference of the eater gene results in a decrease in total sleep (figure 2) due to shorter more frequent sleep episodes during the night (figure 3B-C). Impaired phagocytosis would decrease the concentration of circulating cytokines and muramyl peptides from the degraded bacterial cell walls, both of which have been identified as sleep inducing (Johannsen et al., 1991; Pabst et al., 1999; Stow et al., 2009). These results would suggest that the immune system, specifically phagocytic activity, is indeed playing a role in normal sleep behavior and regulation.

Studies have also discovered that the circadian rhythm is linked to a stronger immune response, with more activity during the night (Kuo et al., 2010; Lee & Edery, 2008; Stone et al., 2012). The circadian clock has been found to regulate cytokines
secretion as well as phagocytosis (Keller et al., 2009; Stone et al., 2012). In particular TNF secretion is found to be higher during the night upon an immune challenge and mice that lack TNF receptors have been found to sleep less during the night (Fang, Wang, & Krueger, 1997; Igaki & Miura, 2014; Igaki et al., 2002; Kauppila et al., 2003). In Drosophila, there is a TNF homolog, Eiger (Igaki & Miura, 2014; Igaki et al., 2002; Kauppila et al., 2003). A reduced concentration of TNF due to impaired phagocytosis could then lead to the reduced sleep that we observe during the night but does not affect day sleep (figure 2).

To gain a better understanding of this mechanism, the levels of Eiger could be measured in flies with impaired phagocytosis. Mutant flies with Eiger knockdown could also be monitored to measure sleep behavior. A better understanding of the immune system’s interaction with sleep could help gain more insight into sleep regulation. Future studies could also focus on the gut microbiota as the source of activation of the immune system in absence of illness.
Chapter V

Conclusion
The importance of the gut microbiota to its host has become more evident in recent years. It has been shown to be able to influence behavior and physiology (Borre et al., 2014; Cryan & Dinan, 2012; Lieve Desbonnet et al., 2015; Diaz Heijtz et al., 2011; Dillon et al., 2000; Ezenwa et al., 2012; Manco, 2012; Sharon et al., 2010; Thaiss et al., 2014). Interestingly, changes in behavior of insects range from swarming patterns of the desert locust to mating preference of the fruit fly, as well as changes in anxiety like behavior changes in mice (Bercik, Collins, & Verdu, 2012; Diaz Heijtz et al., 2011; Dillon et al., 2000; K.-A. M. Neufeld et al., 2011; Sharon et al., 2010). It has also been found that these changes in behavior in mice have been correlated with changes in protein expression of the central nervous system, such as BDNF (Diaz Heijtz et al., 2011; K.-A. M. Neufeld et al., 2011). Changes in these proteins have also been associated with other mental illnesses beside anxiety such as depression, stress, schizophrenia, autism, and Alzheimer's (Berry et al., 2012; Karege et al., 2002; Olsen et al., 2013; Phillips et al., 1991; Shimizu et al., 2003; Taurines et al., 2014; Toyooka et al., 2002). These types of ailments have also been associated with sleep disorder, however a link between the gut microbiota has not been explored.

In this study we aimed to find the effects that sleep may have over the intestinal microbes as well as the effect that the intestinal microbes may have over sleep. Furthermore, seeing as muramyl peptides, fragments of bacterial cell walls, are able to promote sleep and that phagocytic activity of macrophages result in muramyl peptides, we look at phagocytic activity as possible key player in normal sleep regulation. Using 16S amplification and next generation sequencing we studied the effects of sleep on gut microbe quantity and composition. To observe
the effects of the microbes of sleep, flies were treated with a broad spectrum antibiotic cocktail was use to see the effects on sleep. Lastly, macrophage phagocytic activity was impaired and sleep behavior was monitored.

With the use of the next generation sequencing and QIIME analysis of 16S amplicon, we observed changes in total number of sequence read and a tendency for changes in specific bacterial groups. We were able to discern a decrease in the total number of sequence reads in the samples collected during the night as well as in the samples of flies that have been sleep deprived. This could indicate that there is a decrease in bacterial load during the night as well as during sleep lost. These results correlate with findings in fruit flies which indicate that the immune system is in fact more active during the night, in specific macrophage activity, as well as after sleep deprivation (Stone et al., 2012; Williams et al., 2007). The correlations seen in the total number of sequence reads with expected results of bacterial loads and those of other studies could show preliminary indication of changes in total number of microbes. These results could be confirmed by using real time PCR to determine bacterial loads (Nadkarni, Martin, Jacques, & Hunter, 2002; Ott, Musfeldt, Ullmann, Hampe, & Schreiber, 2004). Taken together, these could suggest that sleep is an important part of gut microbiota homeostasis and that the immune system is playing an important role in this regulation. Also change tendency in specific bacterial groups that are not seen in mutant flies that do not compensate for sleep lost could indicate an important role of bacterial groups in sleep behavior and/or that sleep influences the gut microbes. These results could indicate that specific species of bacteria, such as those observed with the Alteromonadaceae family, which show a tendency to increase in sleep deprived flies but
show no change in flies which do not compensate form sleep loss, could indicate that they have a relation to sleep compensation. This bacteria family has been studied in seawater composed of gram negative bacteria of the *Gammaproteobacteria* class (Allers et al., 2007; Ivanova, Flavier, & Christen, 2004). The gram-negative bacteria, from the *Stenotrophomonas* genus, which also belong to the *Gammaproteobacteria* class, show a tendency to decrease in deprived flies. The fact that these tendencies are both from the *Gammaproteobacteria* class and gram-negative require further investigation to elucidate the importance of these characteristics and the role the immune system could be having on the effects observed.

It has been observed that bacterial diversity in insects tends to be lower then in mammals (Dillon & Dillon, 2004; Douglas, 2011; Engel & Moran, 2013; Jones, Sanchez, & Fierer, 2013; Wong et al., 2011). It is thought that the immune system may be playing an important role in this characteristic. Insect only posses an innate immune system while mammals aside from an innate response also have an adaptive system (Mcfall-ngai, 2007). It is believed that the adaptive immune system is able to recognized microbes that are beneficial to the host and in this way is able to mange a more complex microbe composition. However, the innate immune system cannot discriminate among bacteria and will keep a tighter control in general (Douglas, 2011). This small diversity could explain why only small change tendencies are observed in the composition of the flies and would lead us to expect a clearer change in mammals where the diversity is greater and the adaptive immune system could also influence the outcome.

To better understand a possible interaction between sleep and the gut microbiota, we looked to see how the intestinal microbes could affect sleep. For this, a broad-
spectrum antibiotic cocktail treatment was used to disrupt and eliminate the intestinal bacteria. We found that the type of effect on sleep is dependent on the stage in which exposure to antibiotic occurred. Antibiotic treatment during the development showed a decrease in sleep behavior due to shorter sleep episodes and an increase in activity. This type of effect on sleep structure is seen on mice with damage neurons important for sleep regulation (John & Kumar, 1998). However, flies that were exposed to antibiotic only as adults showed an increase in sleep. This could indicate that the presence of the gut microbiota is indeed having an effect on early central nervous system development, in particular those related with sleep behavior. The increase in sleep of the flies that were treated as adults, is similar to that of flies that are fighting off an infection (Kuo et al., 2010). These results could indicate an imbalance in the gut microbes by the treatment of antibiotic that could be provoking an immune response similar to that observe in presence of an infection (Dethlefsen et al., 2008; Kuo et al., 2010; Russell et al., 2012; Willing et al., 2011). To eliminate the antibiotics as the causative of the changes in sleep observed, sleep patterns of axenic flies be recorded and compared to those that have been treated. Axenic flies are reared by collecting fly embryos, surface sterilizing them and transfer them to sterile nutritive medium for continued development. Another possible way of disrupting the gut microbiota is by repeatedly changing the flies to sterile food. This is based on the finding that the fruit fly gut flora is maintained by the constant intake of microbes from the food (Erkosar & Leulier, 2014).

Aside from how the gut microbiota and sleep may be influencing each other, we also looked into the immune system as a possible intermediate between the gut microbes and sleep. First, it is well known that the immune system components, such as pro-
inflammatory cytokines, can promote sleep as well as muramyl peptides as a result of phagocytic activity. However the impact that macrophages could be having on sleep behavior has not been established (Davis & Krueger, 2012; James M Krueger, 1990). Since research has shown that macrophages are more active at night and that they are able to release muramyl peptides and cytokines, both of which have been shown to induce sleep, we looked at the sleep patterns of mutant flies with impaired phagocytic activity (Johannsen et al., 1991; Kocks et al., 2005; Kuo et al., 2010; Stone et al., 2012). We found that macrophage activity is indeed playing a role in normal sleep regulation. A similar sleep pattern as that seen in flies treated with antibiotic during development was observed. If we consider that early antibiotic treatment of flies eliminates or greatly reduces gut microbiota we could deduce that the interaction of the gut microbiota with macrophages could be playing an important role of sleep regulation. We would expect to see these similar sleep patterns when there are no microbes that will activate phagocytic activity in macrophages or if the phagocytic activity is impaired. To gain further insight into this mechanism, real time PCR could be used as a tool to determine the bacterial load in flies that have been treated with antibiotic (to confirm the extent of gut microbiota elimination) as well as the impaired macrophage mutant flies (to determine changes in bacterial load when there is no phagocytic activity).

In conclusion, the results obtained in this study suggest that not only is sleep playing a role in gut microbiota homeostasis but also that the presence of the gut flora is important in normal sleep regulation. We propose, then, that the observed increase in activity of macrophages during the night (due to circadian rhythm) (Stone et al., 2012) leads to a decrease in gut microbe community, which in turn, promotes the release of
somnogenic MPs and cytokines as part of the phagocytic activity (Figure 1A). However, in regards to sleep deprivation, our findings correlate with other studies that have observed that sleep loss seems to provoke higher activity of the immune system and an increase in pro-inflammatory cytokines (M. R. Irwin et al., 2008; M. R. Irwin, Wang, Campomayor, Collado-Hidalgo, & Cole, 2006; Mullington et al., 2009, 2010; Williams et al., 2007). Therefore, we propose that sleep deprivation, by way of an unknown mechanism, is activating macrophages that could lead to a decrease in gut microbiota and as a consequence result in the release of MPs and cytokines (Figure 1B). The increase of these circulating somnogenic molecules would promote sleep behavior and maybe playing a role in sleep compensation after sleep lost. We also show, for the first time, a link between the phagocytic activity of macrophages on normal sleep behavior. The changes in sleep behavior observed in flies treated with antibiotic along with the changes observed in flies with impaired phagocytic activity would suggest that the gut microbiota is linked with sleep behavior by way of the immune system.

However, various factors remain to be established. For example 1) the extent of macrophage activity on the bacterial load or composition of the intestine microbiota 2) determine the bacterial load by real time PCR of flies during the day, night, sleep deprived and non-sleep deprived 3) dissect the mechanisms underlying the differential effects of adult and developmental exposure of antibiotics on sleep behavior 4) investigate the mechanisms of how sleep deprivation and circadian rhythms alters macrophages activity 5) further research into the changes in levels of synaptic protein expression that have been associated with sleep behavior. Our findings could provide an
important focus for the study of sleep problems and the development of treatments by way of probiotics.
Model for macrophage, gut microbes and sleep interaction

Figure 1: (A) Proposed model for macrophage, gut microbes and sleep interaction during normal sleep. The circadian rhythm promotes higher phagocytic activity during the night of the gut microbiota producing in turn muramyl peptides (MPs) and cytokines that can induce sleep. (B) Proposed model for macrophage, gut microbes and sleep...
interaction during sleep deprivation. During sleep lost macrophages are activated by an unknown mechanism resulting in higher immune activity over the gut microbiota. This results in the release of sleep promoting MPs and cytokines by the activated macrophages and in this way play a role in sleep compensation after sleep loss.
References


