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A Video-Tracking Method to Identify and Understand Circadian Patterns in Drosophila Grooming

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A VIDEO-TRACKING METHOD TO IDENTIFY AND UNDERSTAND CIRCADIAN PATTERNS IN DROSOPHILA GROOMING

By

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A VIDEO-TRACKING METHOD TO IDENTIFY AND UNDERSTAND CIRCADIAN PATTERNS IN DROSOPHILA GROOMING

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Grooming is nearly universal in terrestrial animals and is important for animals to maintain body surface condition. In insects, grooming is controlled by relatively simple nervous system, so the study of grooming may reveal basic principles of grooming. While recent progress is being made on understanding the mechanism of stimulated grooming control in *Drosophila* [23, 24], the internal regulation of non-stimulated grooming remains unexplored. It is possible that the circadian clock plays an important role in the internal regulation of grooming. However, a key problem in the study of grooming's circadian control is the difficulty of obtaining and interpreting long-term grooming data.

This thesis focuses on long-term grooming and examines regulation of the behavior by the circadian clock. To quantify and categorize the long-term grooming data I developed a method based on machine learning technology that can identify grooming events automatically from recorded video clips of fly's freely behaving. My research showed that grooming is regulated by the circadian clock and its circadian rhythm is related to the animal's locomotion rhythms. However, I show that grooming does not occur as a direct response to locomotion.

To my beloved friends for their endless love,

Understanding, support and encouragement

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CHAPTER 1

Introduction

1.1 Experimental Motivation

Grooming is broadly defined as a behavior that consists of all forms of body surface care [1]. Most terrestrial animals spend considerable time grooming [2] and this near universality indicates that grooming fulfills an essential role for animals. In different species, grooming assumes different forms: birds preen the oily substance produced by the preening gland on their feather and skin, cats and dogs lick their fur with their tongues, flies sweep their body parts with legs. Despite its different forms, in all cases grooming fulfills the basic function of maintaining a clean body surface. Grooming behavior has been studied for some time, especially in mammals, and these measurements have revealed that the behavior is involved in many more functions, such as maintenance of insulation, thermoregulation, communication or social relationships [3–11].

Although grooming is near universal and is involved in many functions, how it fits into animal's daily schedule has not been fully studied. Time spent on grooming is quite different among different species. For example, male adult rats spend 40% of their waking time in grooming themselves, while spider monkeys spend only 2.5% of their daily activity to grooming [12,13]. Why the amount required for grooming is so diverse remains unknown. It's very likely that all animals spend some time in grooming every day. Some other daily behaviors, such as locomotion, are usually controlled by two different mechanisms. The first one is that the behavior is immediately triggered by

external stimuli, performed as a passive reflex to emergency situations (e.g., "running away from predator" in locomotion behavior). The second one is that the behavior is actively regulated by internal program to fulfills daily demand (e.g., "searching for food" in locomotion behavior) [14]. The control of grooming may also include both internal and external triggers, since grooming is in some way similar to locomotion in that both need the coordination of limbs and body. The regulation of locomotion has been studied and shown to be under control of the circadian clock. By contrast, the regulation of grooming is poorly understood. Considering that grooming plays the role of maintaining a clean body surface, the regulation of grooming may relate to those behaviors such as feeding and locomotion that are likely to cause the surface to accumulate food debris, dirt and microorganisms that need to be removed [15]. Therefore the timing relationship between grooming and other behaviors may help us understand the regulation of grooming. Especially, as the regulations of locomotion and feeding are both under control of the circadian clock [16,17], whether grooming also exhibits a circadian rhythm can reveal the relationship between grooming and the circadian clock.

Drosophila is a good model to use when studying grooming since it is a frequent groomer [18] and has a rich history in neural science, behavior study and disease study [19–21]. The study of *Drosophila* grooming can be traced back to the 1960's [19][22]and progress has been made in understanding how grooming is controlled by a neural system. For example, a recent study has identified neurons that are involve in the process of grooming triggered immediately by dust coating the fly or displacement of antenna [23] [24]. However, most studies on grooming control focus on short-term grooming, which is grooming performed right after a stimulus. Although revealing the mechanism of passive

pattern of grooming, these studies fail to explain grooming events that are not triggered by any apparent stimulus and are not able to reveal the mechanism of internal regulation, thus prompting us to study long-term grooming and how it relates to other daily behaviors, especially locomotion and feeding.

A difficult problem in obtaining long-term grooming data in *Drosophila* is how to identify every grooming event from video. Extracting grooming by watching the video and checking it frame by frame takes too much time and make it impossible to obtain the grooming events over a long period for a considerable number of flies. Recently, video tracking has become a powerful technique to study fly behavior, and some work has been done in automatic detection of fly grooming. However, these methods are hard to apply to the study of long-term grooming due to their limitations. For example, in Kain's [25] method, the preparation of their tracking system is elaborate and time consuming while only one fly can be monitored at a time. Moreover, they require the fly's back to be tethered thus inhibiting its activity. Mendes's work [26] allows the fly to move freely while keeping track of leg movement, but can only last for several seconds because it requires that the insect walks within the narrow field of view of a high-speed camera. As a result of the limitations in existing methods, in most cases, groups must still extract the grooming events by eye [27][28][29]. This constrains the study of grooming to a short duration and limited number of flies.

In this thesis I present a new grooming-detecting method which overcomes the limitations of previous ones. We describe fly behaviors with three features and apply machine-learning techniques to classify behaviors into grooming, locomotion and rest. This new method is able to analyze video recording up to 20 flies for multiple days.

Results indicate that *Drosophila* grooming is actively regulated by the circadian clock. I illustrate how clock genes affect the temporal regulation of grooming by comparing the grooming schedules of different clock gene mutants. I also show that the regulation of grooming is related to the regulation of locomotion, but not related to feeding.

The background of grooming and circadian clock is introduced in Chapter 2. The details of the grooming-tracking method are presented in Chapter 3. The experiments and results are reported in Chapter 4 and 5.

CHAPTER 2

Background of Grooming and Circadian Clock

2.1 The form of grooming in animals

Grooming is believed to be an evolutionarily ancient behavior since it occurs in most animals species [2]. In those species we are more familiar with, that is, including mammals and birds and many insects, grooming can be described as moving the extremities over the body surface and mouthing the body and the extremities. Grooming takes other forms like sand bathing for some birds and mammals [30][31]. For species like fish that do not have extremities or mouths which not flexible enough to reach their body, they rub their bodies over rocks or branches to achieve the same function as the common form of grooming [2]. In social animals, grooming is usually not an individual behavior. Group members help each other to groom the parts which are difficult to reach by themselves, like the area on the back and neck [32]. This grooming between individuals is turned allogrooming, and plays an important role in communication and establishing connections among a group of animals, especially primates.

Time spent on grooming differs a lot between species. Some mammals spend plenty of time on grooming. For example, mice spend almost 40% of their waking time on grooming everyday while other mammals, like spider monkeys, spend only 2% time on grooming [13][33]. In general, terrestrial vertebrates all spend some time on grooming every day.

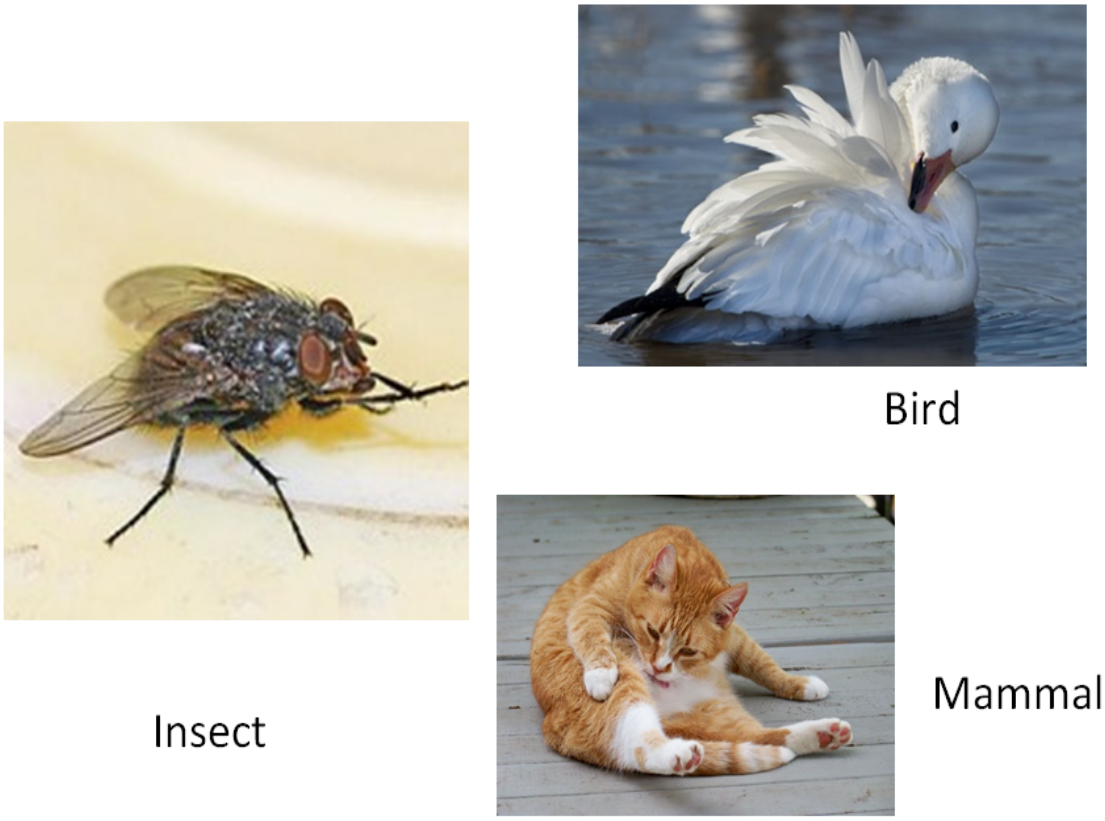

Figure 2.1 Grooming is nearly universal in animals and takes various forms in different species. Flies sweep their bodies with legs and rub their legs against each other. Birds preen feathers with their beaks. Most mammals clean their fur by licking with their tongues. **(**Sources: Google Image**)**

2.2 Function of grooming

2.2.1 Body surface care

The primary function of grooming is to keep the body surface in a good condition. In this respect, grooming may serve as the outer-body functional equivalent of various internal processes, such as the reaction of the immune system and diarrhea, which act to maintain the integrity of the body interior [2].

Although grooming varies in forms among different species, there is one thing that all

grooming forms accomplish----removing foreign objects from their body surface [1,2,15]. For mammals and birds, grooming usually removes dirt, parasite and smooths hair. Besides the hygienic value, grooming is believed to serve other functions. And these functions often relate to the forms of grooming in specific species. For instance, when bird grooming, besides getting rid of dirt and parasites from their feathers, they smear the oily substance secreted from gland on their feather and skin to protect them from water (for water birds) and parasite [34]. Grooming can also help to regulate the body temperature [35]. In rats, self-grooming is important for them to reduce body temperature during heat stress [36]. Grooming spreads saliva over the body and the evaporation of saliva relieves excess heat.

2.2.2 Establishing relationship in social animals

For social animals, grooming has been shown to have greater significance than simply hygienic purposes, especially in primates [37][38]. Social grooming plays a role in reinforcing social structures, constructing family links, and building bonds between group members.

For primates, time spent on grooming is much more than necessary. For example, gelada baboons spend 17% of its waking hours grooming while only 1% would be sufficient to achieve its hygienic need [39]. It has been shown that the act of grooming releases beta-endorphins, which are a natural relaxant, which may indirectly reinforce such social behaviors [40]. In addition, grooming may expose vulnerabilities in the primate, building relationships similarly to humans who undergo self-disclosure.

Figure 2.2 Social grooming is a behavior that members in a group help groom each other's body. Allogrooming refers in particular the grooming between animals of the same species. (Sources: Wikipedia entry "social grooming")

The function of social grooming is supported by the observations showing that the frequencies of social grooming in primates correlate with group size[41]. Members from larger group spend more time on social grooming to bind social group [42]. However, the upper limit grooming time by primate population is 20%, which cannot reach the requirement of predicted social grooming time with the group size of modern people [43] . Language is thought to bridge the gap in maintaining social binding requirement because language allows time to be used more efficiently [43]. In this way, the study on social grooming can gain an insight into when language might have evolved.

2.2.3 Stress and displacement activity

Sometimes grooming behavior may not hold any explicit functions. Many observations indicate that grooming could be induced by stress [44][45]. Animals sometimes perform unnecessary grooming when they face situations that could lead to anxiety. For instance, some hens are trained to eat from a particular food dispenser. When they are hungry and the dispenser is blocked, they often begin to pace and groom themselves intensively [46]. This unnecessary grooming is sometimes considered as one kind of displacement activity which often occurs when an animal is under high motivation for two or more controversial behaviors or combating situation [47]. As a result, displacement activity usually has nothing to do with the competing motivations. Similarly, a human may scratch his or her head or pull their hair when they do not know how to decide or how to solve a problem.

The reason why animals have excessive grooming under stress or when facing conflicting drives is still not clear. One hypothesis is that grooming has an important stress coping function [48][49].When an animal feels stressed but cannot figure out how to get out of the situation which causes the stress, it may groom to relax itself. Disregarding the rationality of this hypothesis, the excessive grooming indicates that the occurrence of grooming does not necessarily have practical functional purpose, providing a new aspect to understand the control of grooming behavior.

2.3 The study of insect grooming

Grooming studies have been helpful for several successful ethological analyze about motivation [50][51][52]. These studies were all about vertebrates. However, the complex

behaviors in insects are especially meaningful to study, for they are controlled by relatively simpler nervous systems. And grooming, is one of the most complex activities that many insects do. Therefore, the study of grooming may reveal basic principles of grooming in insect.

Early studies on insect grooming started from the description and classification of the activity [19]. Insect grooming usually consists of a series of stereotyped movements which are responsible for different parts of body surface (e.g., Figure 2.3 shows the common components of *Drosophila* grooming).

The basic function of insect grooming is to remove foreign objects like debris and parasites from the surface and to keep the sensory organ unaffected by small particles [15]. For instance, observation shows that grooming removes microorganisms from the bases of sensillum (Figure 2.4). It is believed that grooming plays a functional role in insect disease defense [53].

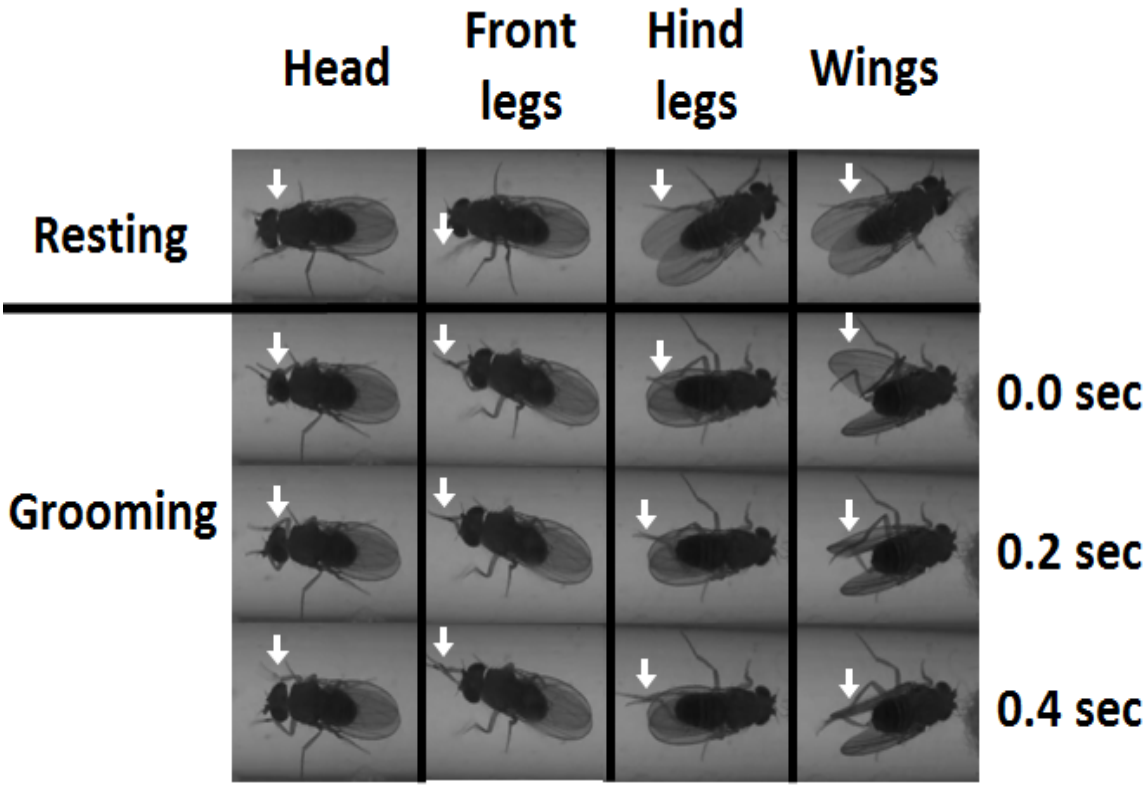

Figure 2.3 Examples of most commonly observed types of grooming. The top row displays postures of a fly in inactive state. The bottom three rows show how its limbs and body coordinate to perform specific grooming movements. Arrows highlight the moving part of fly while grooming. (Sources: Our lab video)

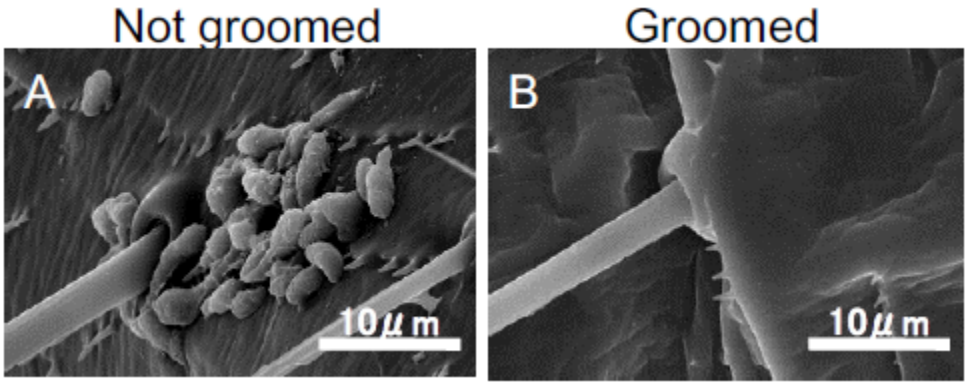

Figure 2.4 from ref. [15] Electron microscope image of before-groomed and after-groomed surface of *Coptoterms formosanus*. (A) The surface are covered with pathogenic conidia before groomed, (B) After-groomed surface have no *condidium*

In recent years, people have been focusing on the control of grooming in *Drosophila* and have made progress on understanding the organization of nervous system related to grooming [24][23]. For example, a neural circuit that can receive the signal indicating antenna displacement and exert antenna grooming events has been identified [23]. Other experiments have shown that dust coating, physical stimulation, or chemical irritants can also induce grooming event [24][28]. Most of these studies were based on the assumption that grooming is induced by the stimulation of the body surface. However, spontaneous grooming without any apparent stimuli has been reported [25,27,54,55]. Insects such as *Drosophila* perform grooming frequently and it is not convincing that these events are induced by any stimulation. The initiation and modulation of these grooming events remains unclear.

It is not yet certain whether both the non-induced grooming and the induced grooming are controlled by the same mechanism or not. When people used dust coating to induce grooming, they found that particles too small are not able to trigger grooming. It is possible that most of foreign objects that attach to the surface were not enough to trigger a grooming event. But it is still grooming's job to eliminate this dirt. It is possible that insects actively regulate grooming according to its "innate" need to keep clean but not due to external stimulation. However, whether grooming is actively regulated or not remains unclear.

To understand the regulation of non-induced grooming, it is necessary to figure out its relationship with other daily activities such as locomotion and feeding. If we could quantify the time and type of every grooming and other possibly relevant events, we may

find out how these behaviors affect each other. However, for insects like *Drosophila*, grooming, locomotion and feeding usually happen many times a day, thus there's little chance to separate each activity clearly and find out the relationship between isolated events. Alternatively, we can look for the pattern of circadian rhythm of each kind of behavior.

2.4 Introduction to Circadian rhythm

The circadian rhythm is an important biological rhythms. The rhythm is cyclic, and has a period of approximately 24 hours [56]. Usually, the circadian rhythm is affected by light. The presence of light, has been shown to reset the cycle, but the exact mechanism is still being explored. One important aspect of the circadian rhythm is a rest-activity cycle. It has been demonstrated that an animal's activity is strongly related to its position in the circadian rhythm [16]. A colloquial example can be found in human activity. Human beings are generally active under light, during the day, and rest under the absence of light, during the night. Of course, the example falls short in modern society where artificial lights can circumvent the natural cycle. On the other hand, bats are nocturnal animals that are active during darkness dawns [57]. They are more active when there is less light and rest during the daytime when there is more light. In other words, the pattern of human activity is converse to that of bats.

However, the amount of light only externally influences the circadian rhythm; it is not absolutely determinative. Even in a room devoid of light, flies exhibit behavior in a approximately 24 hour period [16]. Because there is a free-running rhythm in animals that regulate the animal's brain and regulates all kinds of behaviors. The circadian system

involves in regulating a wide variety of physiological and behavioral rhythms [58][59].

In the laboratory, researchers measure the circadian rhythms in two steps [60]. First, the rhythm is entrained by exposing the animals to 12 hours of light, followed by 12 hours of dark (12:12 LD). Under this LD cycle, behaviors that are under clock regulation synchronize to the light cycle. However, in the absence of entraining, the clock has been found to be variable. Indeed, the period of the circadian clock is determined by a clock gene [16].

Drosophila has been a good model to study the circadian clock [56]. In my work I compared grooming's circadian rhythm with locomotion and feeding to study the control of grooming. In order to present a clearer picture, it is necessary that I introduce some historic researches on the how the circadian clock regulates locomotion and feeding before further elaboration about grooming.

The link between the circadian clock and behavior was first discovered in 1971 by Konopka and Benzer [61]. They found that clock mutants show different locomotion rhythms and arrhythmic behaviors. Besides, in a LD cycle, wild type shows an activity peak around light turning on and another peak around light turning off. Flies tend to become increasingly active several hours before the light transition, indicating that the flies anticipate that light changes periodically.

It is believed that the different parts of the locomotor activity rhythms are regulated by different groups of clock neurons (Figure 2.5) [56]. Clock neurons consist of lateral, dorsal and lateral-posterior neurons. Lateral neurons consist of large-ventrolateral neurons (l-LNvs), five small-ventrolateral neurons (s-LNvs) and dorsolateral neurons

(LNds). 1st -4th s- LNvs regulate the morning activity peak. The LNds and the 5th s-LNv together regulate the evening peak.

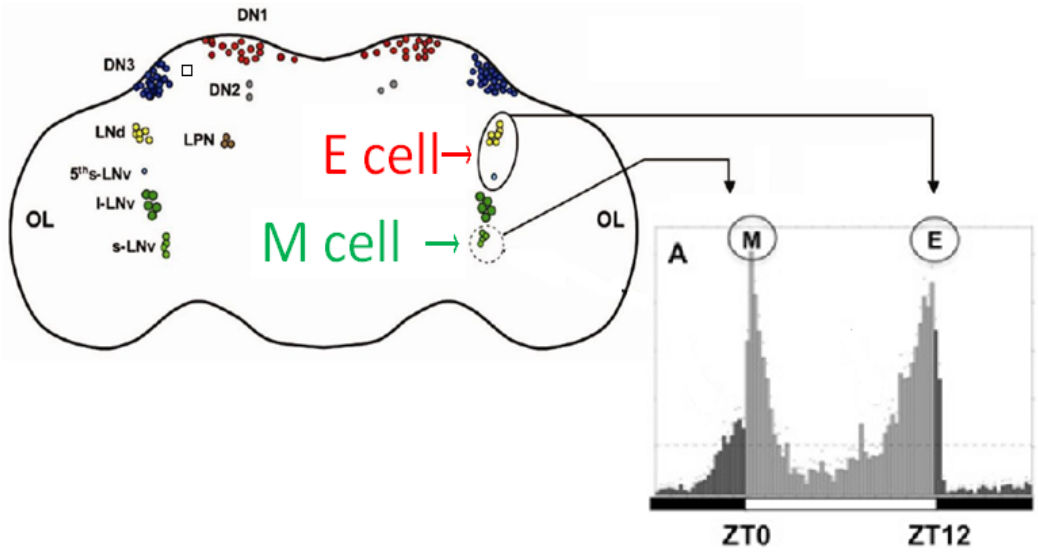

Figure 2.5 adapted from ref. [56]. **Morning and evening peaks of fly locomotor activity are regulated by M-cells and E-cells, respectively.** The three types of brain clock cells are lateral neurons (LNs), dorsal neurons (DNs) and lateral-posterior neurons (LPNs). These cells are responsible for regulating daily locomotor activity rhythms in flies. M-cells that consist of four small-LNv (s-LNv) neurons are required for regulating the morning activity peak in a LD cycle. E-cells that consist of the 5th s-LNv and dorsolateral neurons (LNds) are required for regulating the evening activity peak. OL indicates the location of the optic lobes.

Figure 2.6 shows that the rhythm is regulated by circadian clock. In LD cycle the morning and evening peaks are driven to fit the 24-hr LD cycle. In DD cycle the free-running period is usually different from 24 hr. For example, for flies with short periods the evening activity peak will occur earlier on each successive day in DD (when plotted against a 24 hour time scale, as shown in Figure 2.6), whereas a shift to the right is shown in flies with long periods.

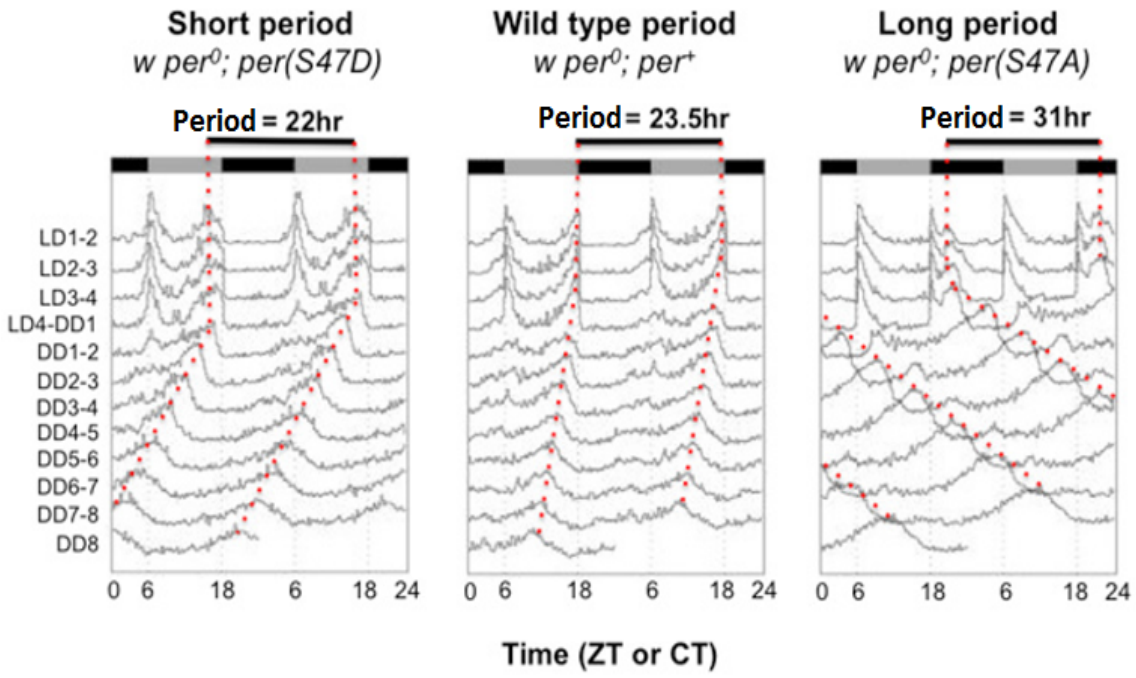

Figure 2.6 from ref.[16] **Double-plot locomotor activity rhythm of flies with wildtype, per^S and per^L flies.** Male flies were entrained for 4 days in LD cycle followed by 8 days DD**.** The red dots mark the evening peak of locomotor activity for each day of the experiments. In LD cycle the activity rhythm is driven by the light so that it fits the 24-hr LD cycle. After transferring to DD cycle, the free-running period is constant and deviates from 24 hr circadian cycle. "ZT" (Zeitgerber time) means that, in an experiment with LD cycle, ZT0 corresponds to light on. "CT" (Circadian time) means that, in an experiment with DD cycle (after several days' LD cycle). CT0 corresponds to the same time as ZT0.

The circadian rhythm of feeding has also been studied and confirmed [17]. Figure 2.7 shows the circadian rhythm of feeding. We can see that the crest in the feeding pattern occurs around light on, and trough occurs around light off, which is different from the locomotor activity pattern.

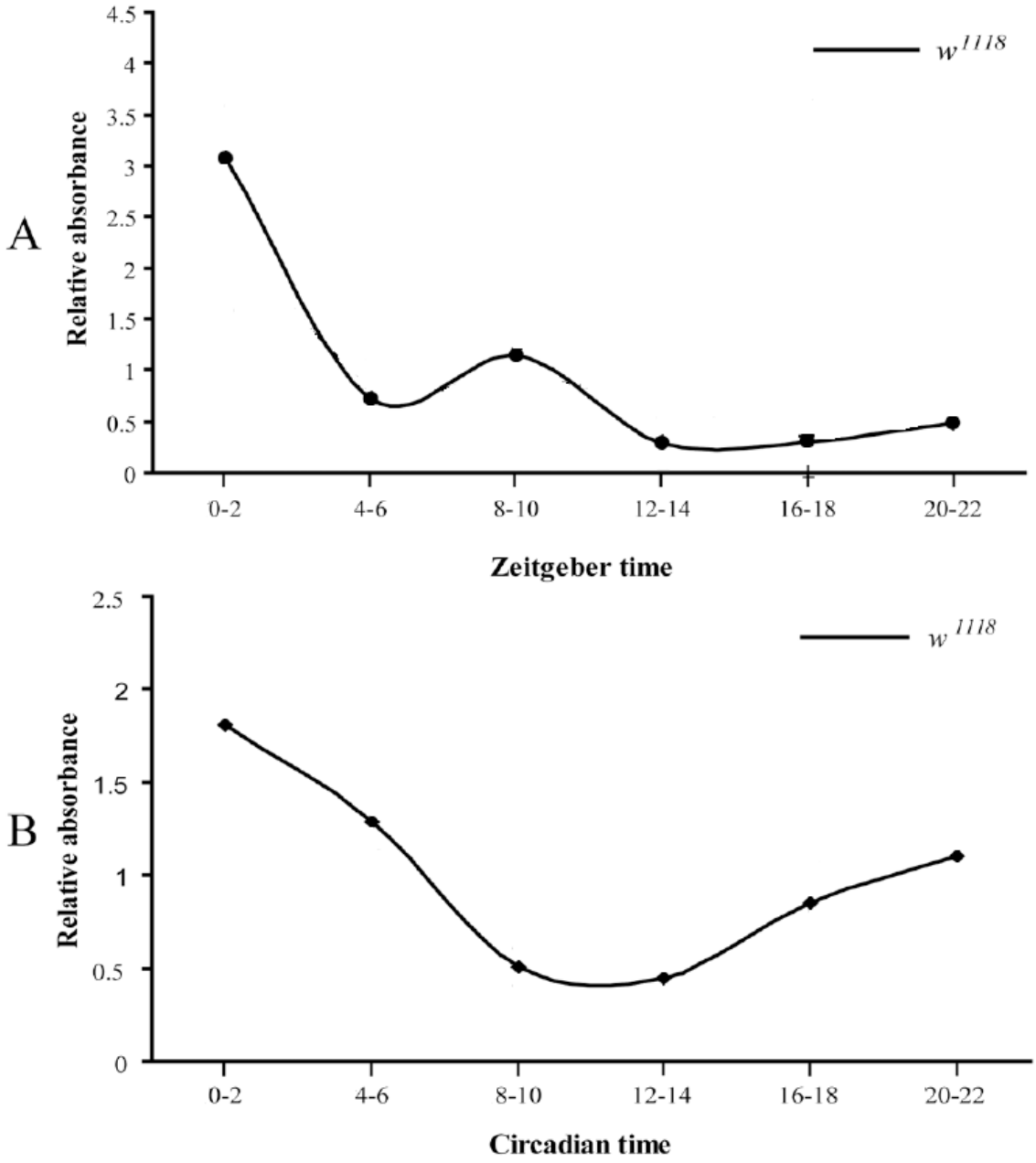

Figure 2.7 from ref. [17]**. Feeding rhythms in LD (upper) and DD (bottom).** Food consumption is measured through the use of a food dye. Flies were fed food for 2 hours at six different times of day. Relative absorbance is the food consumption amount compared to the average amount of all the six times. In LD cycle, feeding shows a crest in the early morning and a trough at night. In DD cycle, feeding shows a peak started in the late night (CT4). For "Zeitgeber time" and "Circadian time", see the caption of Figure 2.6.

Grooming, as a daily behavior, is possible to exhibit circadian rhythm. If it does, we can expect that grooming's rhythm shows regular crests and troughs at certain times.

Some observations in mammals indicate that the occurrence of grooming is often linked to feeding. For example in spider monkey, their daily grooming distribution shows two peaks which coincide with the long resting periods that usually follow the long feeding bouts [13]. Observations of rats, indicates that there is an increasing incidence of grooming activities among the rats after eating [33]. Animals may clean themselves after feeding, probably to remove the food debris from their body surface. Similarly, *Drosophila*'s grooming would be expected to show a peak after the feeding peak. Meanwhile, locomotion may also contribute to the accumulation of small particles on the surface, thus the pattern of grooming is also likely to be affected by locomotion. Yet, the extent of their relation still needs to be studied.

Now, let us come to the regulation of grooming. To do the study in detail, we need to discern the patterns of grooming, locomotion and feeding. However, identifying specific behaviors, especially grooming, is very time and energy consuming work if done by eye. Although with the help video recording, the work could be divided and accomplished by a group of people, the data of grooming is still limited in time and quantity of the sample animals. To break through the limitation and expand the research scale, we need to develop an approach with the help of modern technology by introducing an automatic tracking system. In next chapter I will present the method I developed to identify grooming by computer.

CHAPTER 3

Method to Track Grooming

The basic task in studying grooming is to find out when grooming happens. Before video recording was widely used, grooming was measured with a serial time-event recording device [22]. The experimenter recorded the starting and finishing time of a grooming event by depressing a button on a finger panel. This live-mode recording has limited accuracy since the person watching the fly could not verify that a grooming event was happening until this behavior had been performed for maybe half a second. The application of video recording has made a contribution in identifying behavior since the experimenter can replay the video and has made it much more accurate to determine the starting and finishing time.

In the previous study of grooming, people identified grooming events by manually scanning the video [23,24,27,28]. This method is sufficient for studies of short duration, since their recording time is generally less than one hour. However, for the study of circadian rhythm, the video can be several days long, and thus costs too much time for researchers to identify every grooming event, especially when multiple flies are being studied. Consider an eight-day-long video of 10 flies, theoretically one person needs to spend at least 80 days to identify all the grooming events. To solve this problem, I developed a computer program to do the identifying work. In this chapter I will introduce some basic ideas of video-tracking and present our method which is able to automatically identify grooming events.

3.1 Overview

Computer analysis of *Drosophila* behavior has been studied for years. In general, the task is to extract information people want from images. Every image is recorded as a two-dimensional matrix (suppose the image is black and white) in which every element represents the grayscale value (brightness) of a pixel on this image. Figure 3.1 shows an example of an image and its grayscale value in colors.

So far the program has been able to track the position of the fly, and to calculate the axis of the body and wings. With this information, it is not hard for researchers to analyze the body and wing movements. However, it is not enough to identify grooming only by body and wing movements, To gain a comprehensive picture of grooming, researchers also require information about leg movement, and more precisely, the coordination of legs and body.

Compared to body and wings tracking, leg tracking from images is much more difficult because it requires recognition of specific shapes. Hence, people did some additional work to track the legs, such as marking legs with dye and then tethering the fly on a walking ball [25]. Although the improved method works well to locate the legs and analyze grooming, it has excessive restraints on the fly and the hardware of the tracking system is too complicated to apply on multiple flies.

My method detects grooming only depending on image processing and analysis without any additional disturbance to the fly, so the fly can walk freely in tubes. This method does not aim to find the spatial location of each leg. The idea is to use the total

moving amount of the fly's periphery (generally including limbs and wings) and core (chest and abdomen) to identify the mode of movement. This chapter will introduce the data acquisition system and details of the software's working principles.

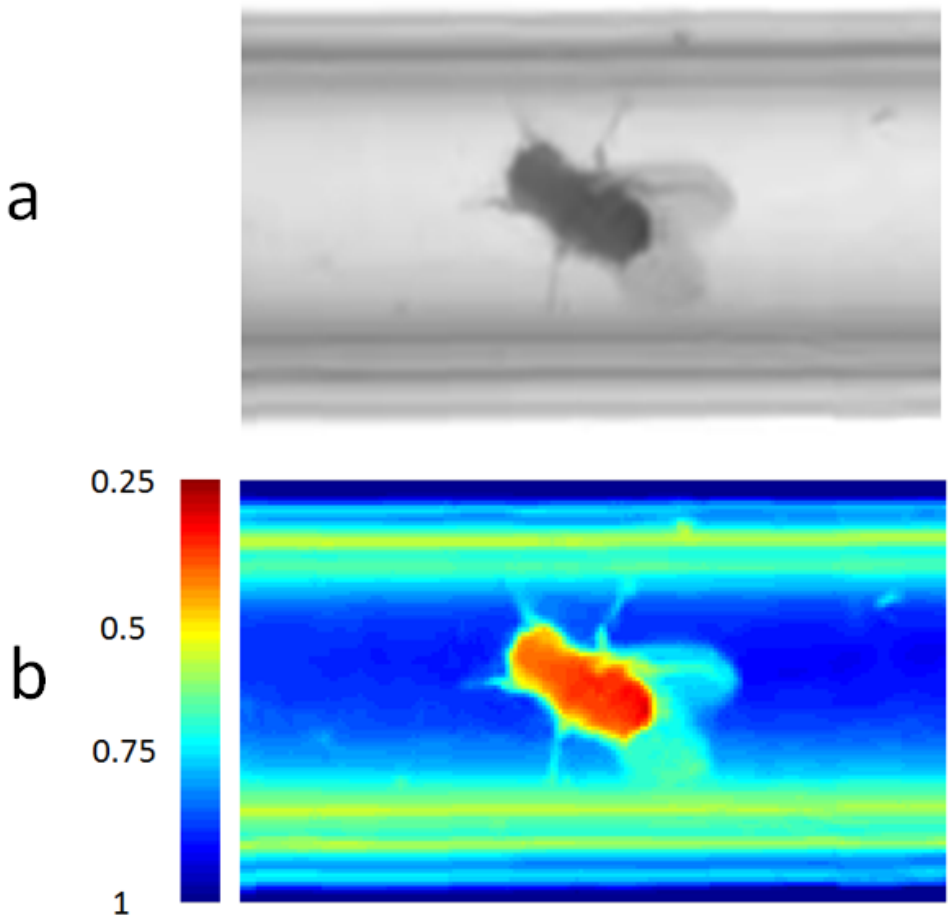

Figure 3.1 (a) An example of fly image. (b) Color shows the grayscale value of every pixel on image (a). The grayscale values range from 0 (black) to 1 (white).

3.2 Data acquisition system

Our data acquisition system is mainly designed to acquire images of multiple flies in tubes over several days. In order to meet this demand, the system should be able to run for a long period of time. The data acquisition system is shown in Figure 3.2. We built a cuboid transparent chamber to hold the tubes carrying the flies. The chamber is supported

by four poles which are fixed on a table to make sure that the chamber keeps steady. Flies are illuminated by an infrared light source from the bottom, which helps to record images in the dark. White LED are placed around the chamber and are controlled by computer to create different lighting conditions. A digital camera (details in Figure 3.2) aplaced on top of the chamber records all the flies and saves the video into the computer.

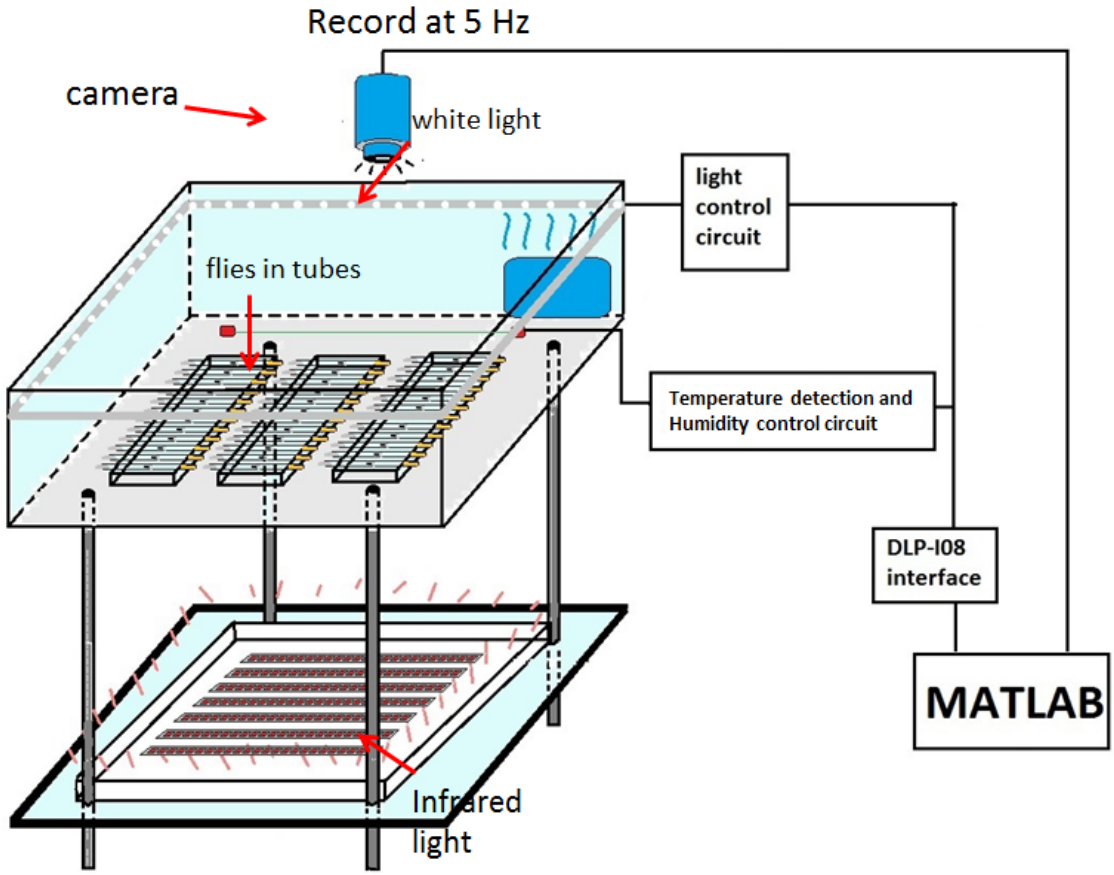

Figure 3.2 Data acquisition system. We used a CCD monochrome camera (The Imaging Source, DMK-23U445) and a vari-focal lens (The Image Source, T2Z-3514-CS) to take video. In order to avoid the influence of light/dark condition on the quality of video, we put a 780nm long pass filter (Midopt, LP780-30.5) in front of the lens. Videos were saved in .avi format with 1280*960 resolutions at 5 Hz.

Each fly is constrained in individual tubes filled with food at one side and cotton on the other side. Usually flies could live for at least two weeks in the tube without any additional maintenance from outside.

The humidity in the chamber is controlled by a wet sponge and several small holes (diameter: 7 mm) on the chamber. The sponge could increase the humidity in the chamber and the hole could decrease the humidity. Thus we could adjust the humidity in the chamber by changing the number of the holes that are open to the space outside the chamber. A humidity detector reports current humidity to the computer every minute as well as the temperature detector. All experiments were done with 5-8 days old male flies at 25-26°C and 70-80% relative humidity.

To track grooming, leg movements must be clearly visible from the video. This requires sufficient resolution for each fly. Though we want to record as many flies as possible at the same time, the more flies we put under the camera the lower resolution it become for each fly. After adjusting a balance between number of flies and resolution for each fly, we finally determined to put 20 flies in the chamber at one time. The raw image from the system is shown in Figure 3.3.

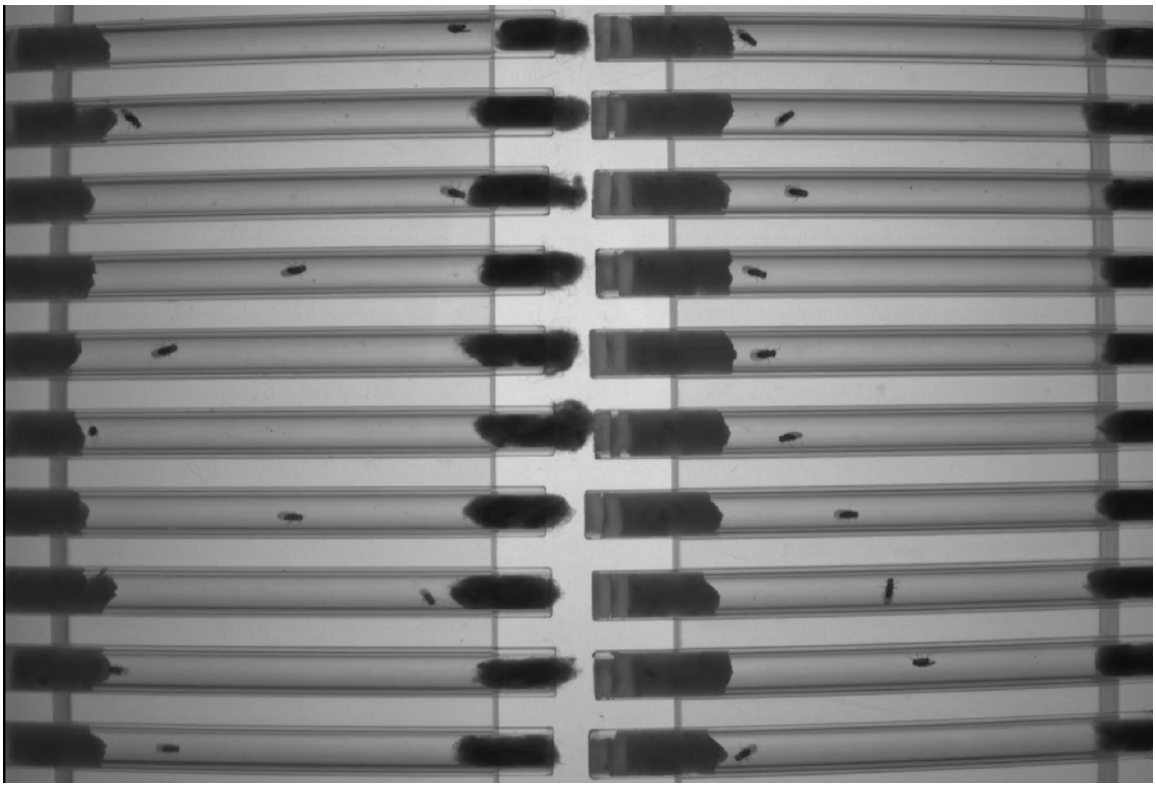

Figure 3.3 Raw image from the data acquisition. Tubes are placed parallel to each other. The left end of the tube is filled with food and sealed with wax. The right end is filled with cotton. Flies can walk freely in the tube.

Preprocessing video and feature extraction

The video analysis takes four major steps using the software: extracting fly's shape, calculating features of behavior, training of the classifier, and classifying behavior. Fly extraction selects all pixels which belong to the shape of fly. The centroid position of the fly is then calculated based on the locations of these pixels. Next, these selected pixels are sorted into two types: periphery and core, and the amount of motion of the periphery and core are calculated separately. With the movements of center, periphery and core of some frames in which the behavior of the fly is known, we train a classifier to sort all behaviors into three types: locomotion, grooming and rest. The detailed procedures will be shown in the following sections.

3.3 Background subtraction

To acquire the target fly's shape from the image, we applied background subtraction. Background subtraction, is a technique in image processing and computer vision wherein an image's particular object is extracted for further processing [62]. Generally, region of interest in an image are objects (flies, in our case) in its foreground. Background subtraction is widely used in identifying and locating moving objects in videos recorded by static cameras. The method is to detect moving objects by differentiating the target frame and a reference frame. The reference frame is often called "background image", or "background".

Due to the fly's movements and daily routines such as eating food and walking around in the tube, the background changes slowly over time. As a result, the background needs to be regularly updated during the long-term video so as to adapt to any non-fly related changes in the settings such as the reduction of food and displacement of food debris. We set every 5000 frames as a video section (1000 seconds long) and one background is made for each section.

There are several common algorithms to make a background image, but here we present our own way to make it, which suits our videos better.

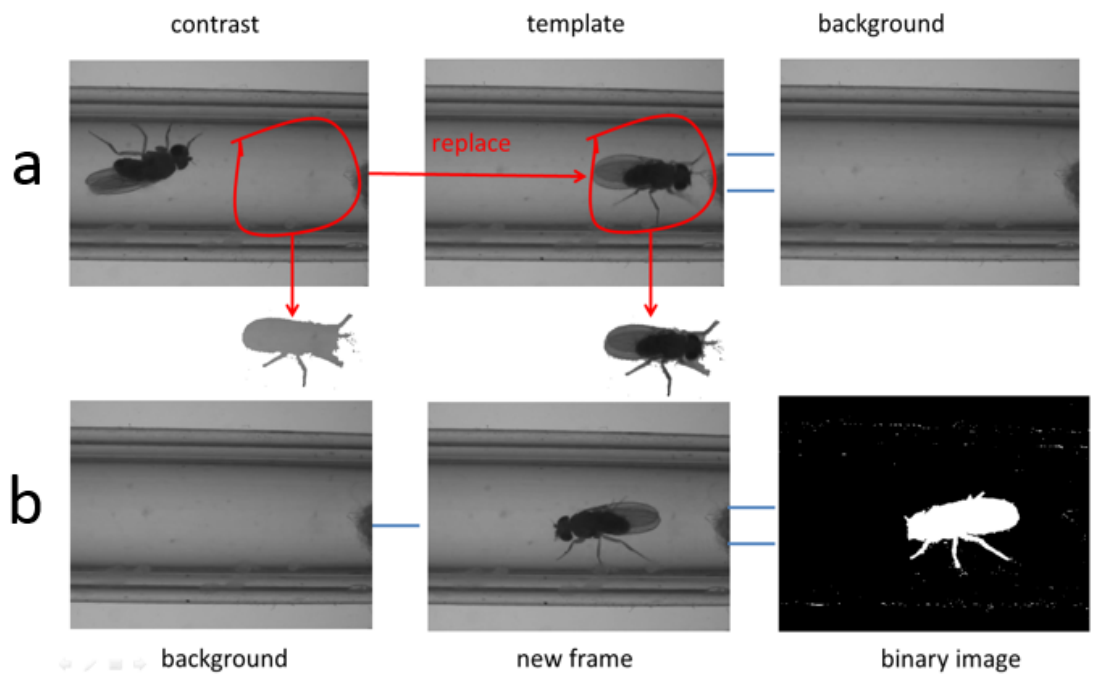

Figure 3.4 Illustration of background subtraction. (a) Compare two different frames to replace fly's pixels with tube's pixels. (b) The different pixels between the background and a new frame are selected into a binary image

3.3.1 Setting up background image

We constructed a background by comparing two frames in the video to erase the presence of fly. We picked the first frame in the video section to be the so-called "template frame" and compare it to another frame in this section called "contrast frame". Flies in template and contrast frames are presumably located at different places so we can find and erase the fly in template frame. Specifically, every pixel in the template frame is compared with the pixel at the same position on contrast frame. If the pixel on the template frame is darker than that of the contrast frame, it is very possible that the pixel on the template frame belongs to the fly. Therefore, we used the grayscale value of the pixel in the contrast frame to replace the pixel on the template frame. However, the difference shown on the pixel may also come from the CCD's noise and fluctuations of

the recording system. As a result, images of a static scene may show different grayscale values on the same pixel, which affects the quality of the background. Thus, it is necessary to set a threshold C_0 as a gap to ensure the difference between the template frame and the contrast frame is from the fly instead of image noise.

"I" is used to represent the image's matrix. "I(x,y)" represent the grayscale value of the pixel locates on (x,y) in the image. Specifically, for a pixel (x, y), if the difference of grayscale value between contrast and template frames is greater than a threshold C_0:

$$I_{template(x,y)} < I_{contrast(x,y)} - C_0$$

Then grayscale value of this pixel in the template is set equal to that in the contrast:

$$I_{template(x,y)} = I_{contrast(x,y)} .$$

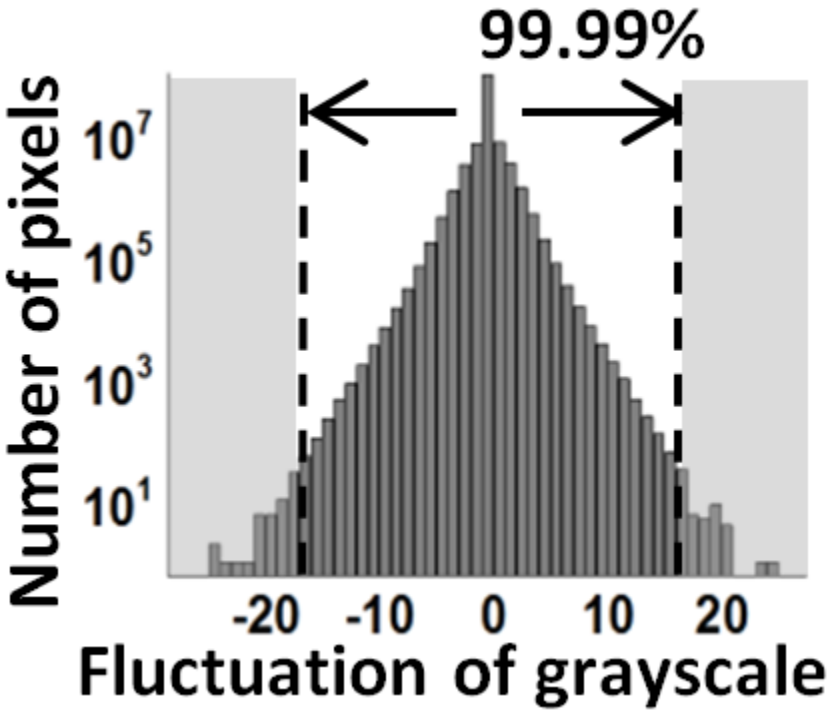

Figure 3.5 The distribution of grayscale value fluctuations in the recording system in the absence of fly movement (died fly in tube). 30 frames are randomly selected from the video and randomly matched into 15 pairs. Within the paired images,

grayscale value of pixels on one image is subtracted by the grayscale value of pixels on the other image with the same location. The subtracted results represent the fluctuation in the recording system. The distribution of all the subtracted results is calculated. A cutoff of grayscale threshold of 15 removes > 99.99% of fluctuations.

While increasing threshold C_0 blocks more noise, it also results in missing some pixels that belong to flies. To obtain the optimum C_0 we tested the image's noise by analyzing a three-hour video of immobilized flies. Without noise, a fixed pixel in every frame should have the same grayscale value. In the test, 30 pairs of frames were randomly chosen from the video and the difference of all grayscale values were calculated and recorded into a matrix which reflects the intensity of fluctuations from the recording system. The distribution of fluctuation values is shown in (Figure 3.5). And finally we set C_0=15, which excludes 99.99% pixels whose grayscale value differences between frames are caused by image's noise.

A common problem in the comparison step is that when a fly in template and contrast frames occupies the same area, so the overlapping area cannot be erased from the template. In order to solve this problem, we used multiple contrast frames to avoid overlapping. For instance, suppose the fly is active in ten minutes, in which time we uniformly pick ten frames, then we can almost be sure that the fly images in these frames will not share an overlapping area. Furthermore, the situation that overlapping area still exists in all the ten frames could result in failure of setting a qualified background. But this "unlucky" situation in constructing background also indicates that the fly does not significantly move during the ten minutes, except in the rare case that despite the movements, the fly coincidentally returned to the same place as it started at the two time point when the contrast frames are taken. For every video section, we picked seven

consecutive frames as contrast frames, and after all the comparison the template frame becomes the background frame for the section.

3.3.2 Extracting the shape of flies

We next compared a frame containing flies with the background frame to extract the fly's shape. If the intensity of one pixel of the new frame is larger than that of the background frame, the pixel on the new frame belongs to the flies. When comparing grayscale value, threshold C_1 is applied to limit the effects of image noise just like when we were creating background.

Select fly pixels: if the grayscale value of a pixel (x, y) is C_1 less than that of the background frame:

$$I_{frame(x,y)} < I_{background(x,y)} - C_1$$

It is selected as a fly pixel and recorded in a binary image "fly" as fly(x, y) = 1(white), otherwise fly(x, y) =0 (black)

Similar to setting C_0, we set threshold C_1=15, which excludes 99.99% of pixels that the difference between the current frame and background are due to noise. Although some noises still remains after applying C_1, these noise pixels are scattered in the frame and do not form a closed object.

Thus, we constructed a binary image "fly". As threshold C_1 ensures that the noise-formed objects are much smaller than fly, we then erase all objects that are smaller than 5 pixel×pixel area(shown in figure 3.7), leaving only the silhouette of flies (Figure 3.7).

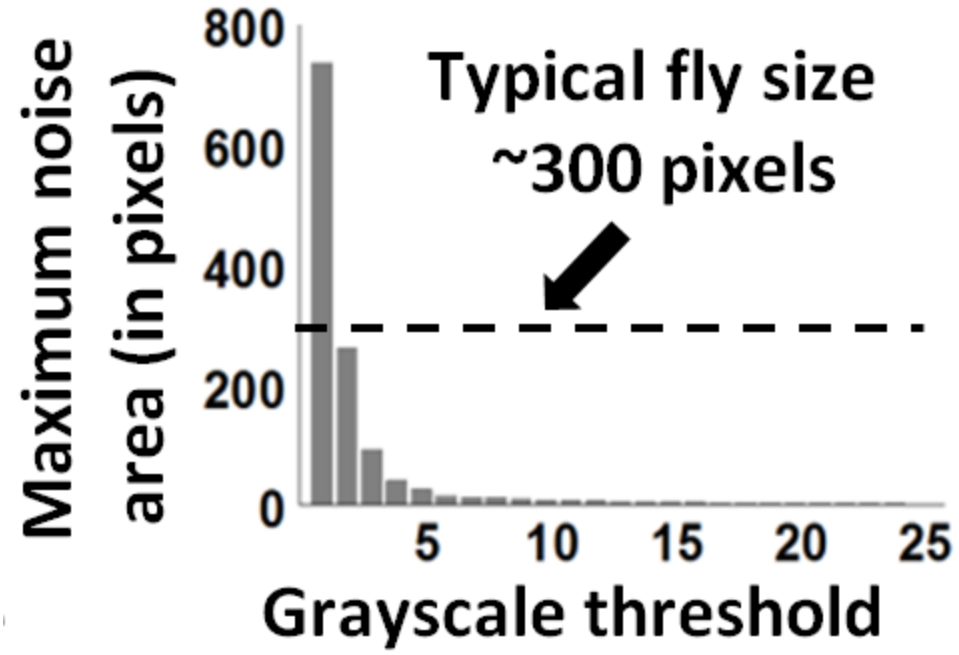

Figure 3.6 Maximum area (pixel×pixel) of a closed object generated by noise when different thresholds are applied to images. From these data, we adopt a value of 15 for parameter C_1 and 5 for parameter S_1

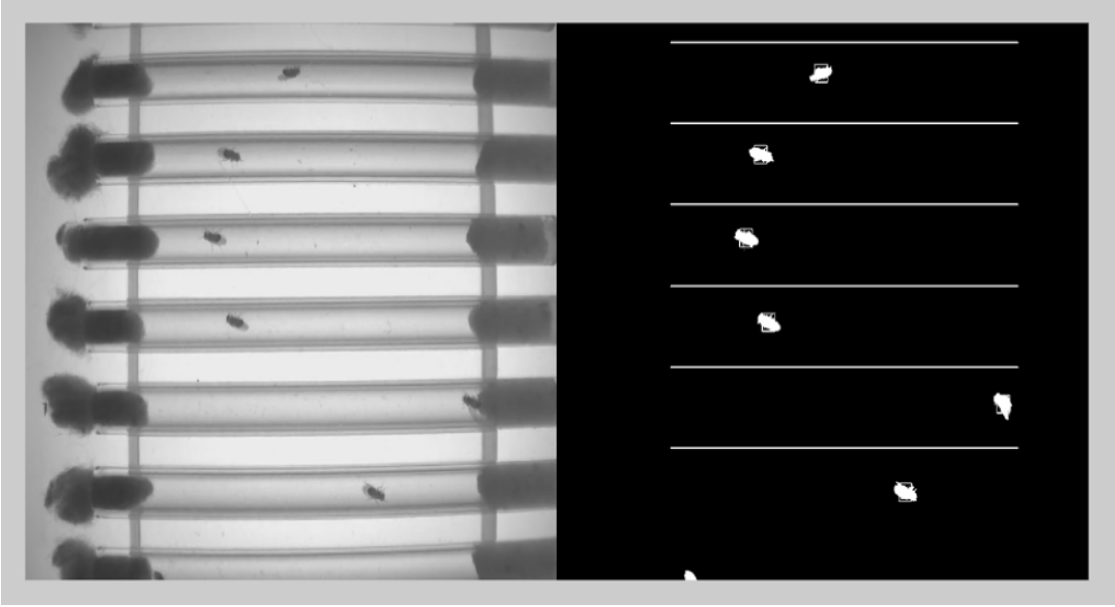

Figure 3.7 Flies' shapes are found after background extraction. The right picture shows that each fly is processed in its own cubicle so the extraction is independent for each fly.

Since the process is independent for each fly (Figure 3.7), in the following section we only talk about one fly in each frame instead of multiple flies, for simplicity.

Now that the silhouette is extracted, we need to calculate the features of behavior for each frame.

3.4 Features to describe and identify behavior

What are features?

Feature is represented by a number which can reflect status of specific behavior. We can use different features to describe animal behavior depending on what type of behavior we are trying to identify. For example, we cannot see a cat but we are told it is moving faster than a certain speed, then we can infer that the cat is probably running, so speed could be a good feature for identifying running. This example shows that a feature to identify specific behavior should be able to distinguish the target behavior and others. When the target behavior is too complicated to be described by single feature, we then use multiple features to describe it.

Types of behavior

In our work we classified all behaviors into three types: (1) grooming (2) locomotion, (3) resting. Grooming is defined as fly rubbing its legs against another part of its body. Locomotion is defined as flies walking in the tube. Resting is when the flies are in a relatively static situation, such as when they stay still and with no activity. Behaviors other than these three types are all grouped into locomotion.

Since grooming is such a subtle behavior which we aimed to identify, the features chosen to describe behavior should be able to reflect the coordination of body, limbs and wings. Notice that when a fly is grooming, its core part does not move much but its legs or wings do. We separated the fly's shape into core and periphery and calculated the movements of the two parts separately. Since Figure 3.1 shows that the area brighter than yellow is core, darker than yellow is periphery, we can use a certain brightness (let's say yellow) to separate the fly. Figure 3.8 shows how these two features perform differently in three types of behaviors: (1) in rest neither periphery nor core has movement; (2) in locomotion both the two parts have significant movements; (3)in grooming, periphery moves much more than core. To quantify the core and periphery movements we defined two features as (1) number of core pixels that are different from previous frame (**CM,** short for core movement), and (2) number of periphery pixels that are different from previous frame(**PM,** short for periphery movement). To achieve a more accurate result, we chose centroid displacement (**CD,** short for centroid displacement) as the third features. The deriving of three features is explained in the following paragraphs.

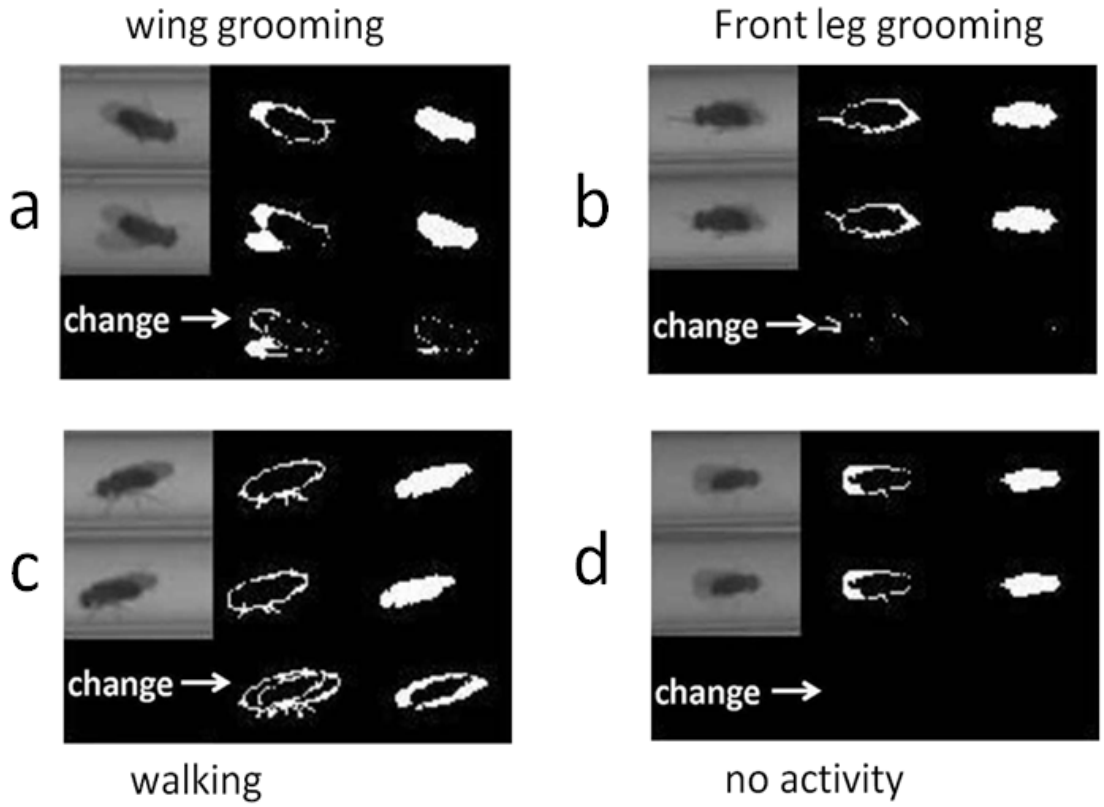

Figure 3.8 Examples of original and processed images of different behaviors: (a) front leg grooming, (b) wing grooming, (c) resting and (d) locomotion. Raw images of one fly in two neighboring frames are displayed on the left row of each panel, followed by its periphery shape in the middle and core shape on the right. Changes in periphery and core are presented in the last row.

Splitting fly into core and periphery

In the classification, the relationship between CM and PM are crucial. As a result, when splitting flies we try to make core and periphery have the same size.

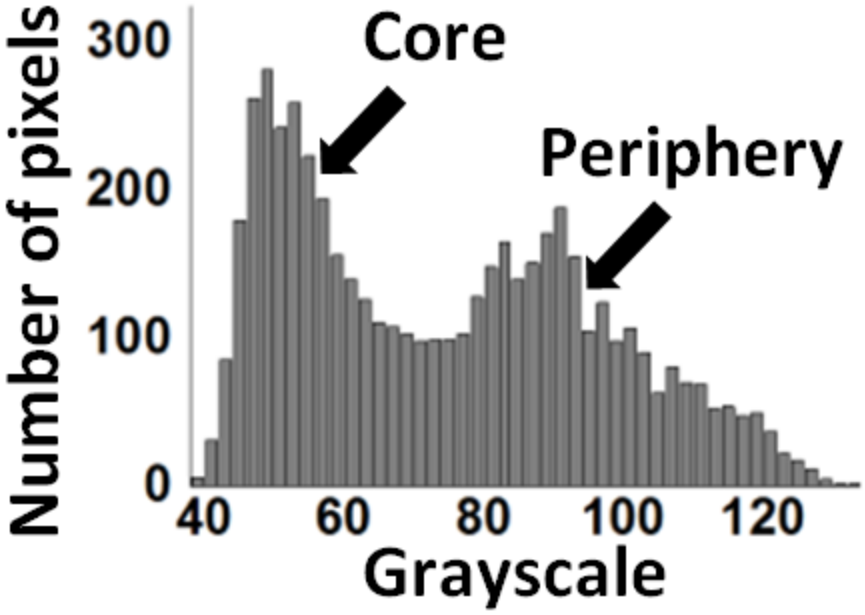

Figure 3.9 Distribution of grayscale values of pixels belonging to 20 individual flies. Two regions are clearly seen: the left region with peak ~ 50 represents the core, and the right region with peak ~ 90 represents the periphery.

We use an intermediate grayscale value to split the fly. The intermediate value depends on the grayscale distribution of each fly. However, the grayscale distribution varies from fly to fly, since the light condition changes across the arena. As a result, the intermediate value for each fly is different. In order to calculate the intermediate value, we first need to determine the average grayscale value μ and standard deviation б for each fly as follows.

Suppose there are n pixels (x_1,y_1), (x_2,y_2), … (x_n, y_n) satisfied by $fly(x_i,y_i) = 1$. The average grayscale value of the fly can be calculated by:

$$\mu = \frac{\sum_{i=1}^{n} I_{frame(x_i,y_i)}}{n}$$

And standard deviation:

$$б = \frac{\sum_{i=1}^{n} (I_{frame(x_i,y_i)} - \mu)^2}{n}$$

Select core pixels:

Given the fact that the core part is generally darker than a certain grayscale value related to μ, the pixels (x,y) that satisfy the condition below will be selected as core pixels, recorded as core(x, y) = 1, otherwise core(x,y) = 0.

$$\frac{I_{frame(x,y)} - \mu}{б} > C_2$$

Select periphery pixels:

The pixels (x,y) fulfilling following condition will be selected as periphery pixels, recorded as periphery(x,y) = 1, otherwise periphery(x,y) = 0.

$$\frac{I_{frame(x,y)} - \mu}{б} \leq C_2$$

To equally divide the fly into periphery and core, we adopted a value of 0.6 for parameter C_2 .

Next, we calculate the number of core pixels that are distinguished from previous frame (**CM**). CM reflects the amount of movement of core during two frames. This is quantified as the number of pixels that are not in the overlapping area of the core part in the two neighboring frames.

$$core(x, y, t) \neq core(x, y, t-1)$$

The pixels which are not in the overlapping area are recorded as difcore(x ,y, t) = 1, and the total number of pixels which shows core movements is calculated as:

$$CM(t) = \sum \big(difcore(x, y, t)\big)$$

The number of periphery pixels that are different from previous frame (**PM**) is calculated similarly.

Centroid position

Centroid movement (CD) is referred to as the centroid position's difference from previous frame. We calculate the position of the fly centroid from the binary image. Suppose (x_1,y_1), (x_2,y_2), … (x_n,y_n) are all the points satisfied by $fly(x_i,y_i) = 1$. The fly's position can be calculated from:

$$(x, y) = \frac{\sum_{i=1}^{n}(x_i, y_i)}{n}$$

As the tube is approximately one dimensional, when calculating centroid movement we ignored movement perpendicular to the tube.

3.5 KNN algorithm for classifying behavior

With the features of every frame, it's still difficult to simply set individual thresholds for each feature to classify behaviors since these features are not independent. This is because the differences among behaviors are not only reflected on the magnitude of each feature but also on the relationship between features. To solve the problem we applied K-nearest-neighbor (KNN) algorithm to classify fly behavior for each frame in the video [62]. The KNN method works by placing an unclassified data point into a space in which the

rest data points in the space are classified (known as training set) [63]. The space is called feature space and in our algorithm it represents CD, CM and PM on its three axes (Figure 3.11).

Figure 3.10 illustrates how the KNN classifier works on a two-dimensional feature plane for simplicity. The two axes of the plane are CM and PM. First we manually pick out a certain number of frames in which the fly is grooming, calculate the value of CM and PM of each frame and then map them into the feature plane. These points represent some grooming examples, shown in red. Then we do the same thing for locomotion and resting, as green and blue, so that each type of behavior have some example points on the feature plane, and they are generally located in different areas. For example, the CM and PM of blue points (resting) are normally very small since neither core nor periphery move during resting, so the blue area is the closest one to the origin. The red area (grooming) is closer to x axis than the green area (locomotion) since the core movement is smaller in grooming than in locomotion. Ideally, different areas are completely separated, but in practice, overlapping is unavoidable. If those areas of different color overlap too much, it means that the features we choose are not good enough to classify behavior.

Now we have a new frame in which we do not know yet what the fly is doing, we can also map it into the feature plane, shown as a black star point. Now we try to figure out which color this new point most likely belong to. We find the new point's 5 nearest sample points (suppose we set K=5) and figure out what color the 5 sample points share

most. That color will be marked on the new point, which means we classify that frame into a certain type of behavior.

The real classifier is shown in Figure 3.11. For each class, 18000 training points are loaded into the feature space. K=10 is settled to balance the calculation time cost and accuracy.

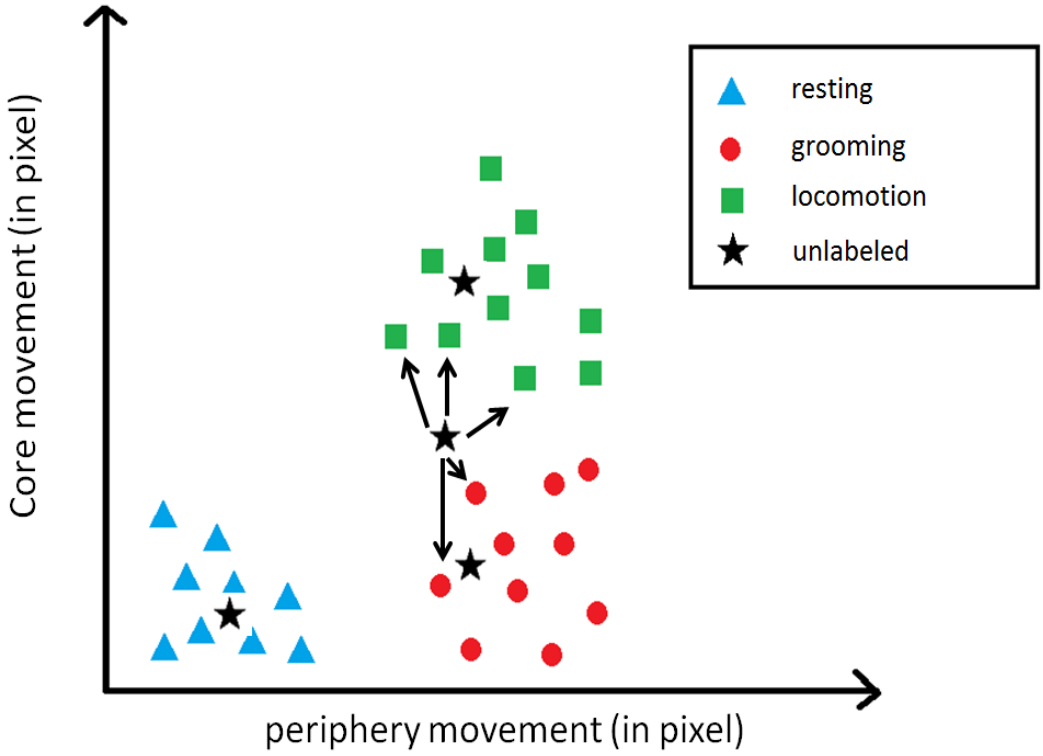

Figure 3.10 Illustration of how the KNN algorithm works on a two-dimensional feature plane. Colored points represent the training set and black points represent unlabeled points. In this figure K=5.

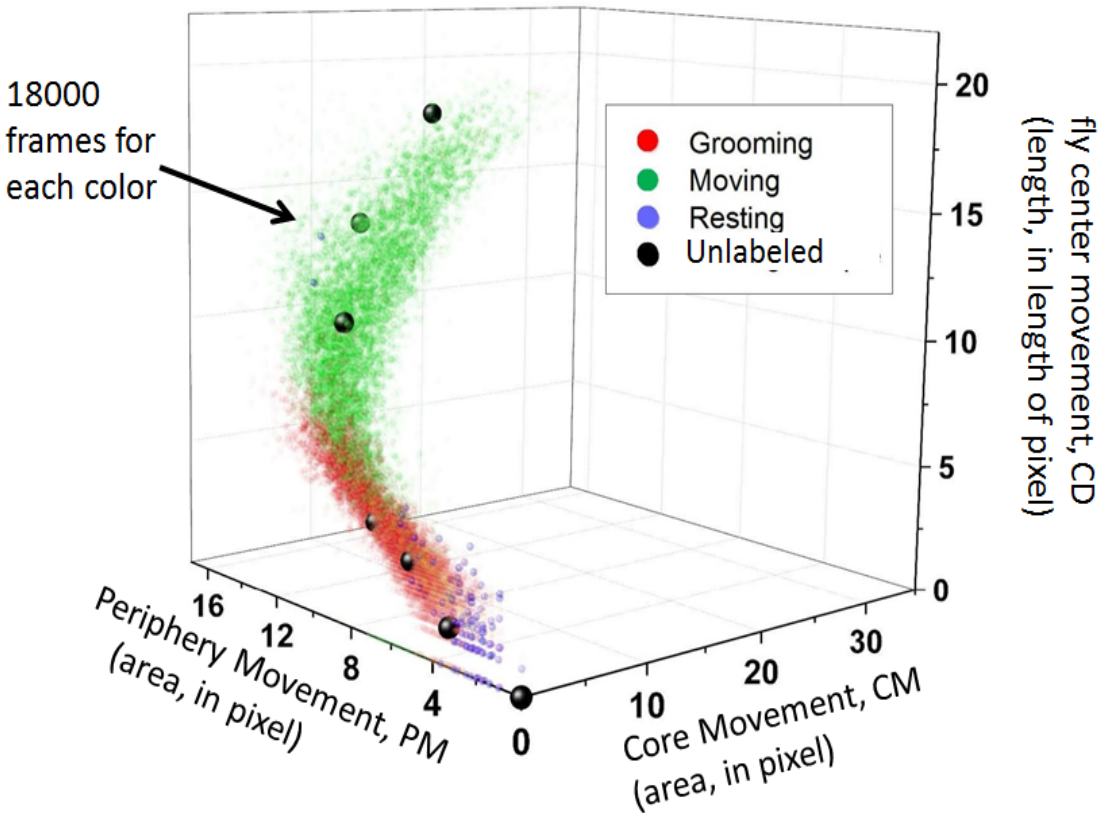

Figure 3.11 The classifier in our method. The K-nearest-neighbor algorithm works by placing an unclassified data point (representing a frame) into a feature space with pre-classified (shown in different color, the training set). The three axes of the feature space are the changes in fly periphery (PM), core (CM), and center position (CD). Fly activity in the feature space is separated into three regions: grooming (red), locomotion (green) and resting (blue). A new (black) frame is classified by its location in this space. Training samples (N=18000 for each color) and 9 testing points in PM-CM-CD space are shown.

3.6 Grooming filter

There are two reasons that we need a grooming filter step after gaining the classification of all the frames from the classifier.

First, when picking out the grooming frame as the training set, it is sometimes hard to tell whether a single scratch which last for very short time (less than a second) should be

incorporated into grooming. This inevitable ambiguity is due to a high level of plasticity in grooming-related behaviors and often-subjective observation of behavior itself. In this work, events that are too short are not identified as grooming for simplicity. Based on our experience of observing the flies in the videos, most grooming events last longer than three seconds. So we only looked for grooming longer than three seconds when selecting frames for training data. For the same reason, the output of every grooming event should also be restricted to those events that are longer than three seconds. One of the purposes of the filter is to make sure that all the final grooming events have adequate length for our study.

Second, there are inevitable false classifications in a series of "grooming" frames. In the classifier, different classes of behavior have overlapping region. Although the KNN algorithm would assign the point to one of the classes, it may still be incorrect. For example, a grooming frame might be classified as locomotion, or vice versa. So, for a piece of grooming which lasts multiple frames, some may be put into wrong classes. Similarly, in a piece of locomotion, some of the frames may be classified as grooming. The cause is that the feature's value is dependent on the difference of the fly's postures between adjacent frames. Yet, during an event, the behavior might not be consistent enough so that the features in this frame do not represent the typical value of this behavior. In this case, we need to allow that not all frames in a piece of grooming event are labeled as "grooming".

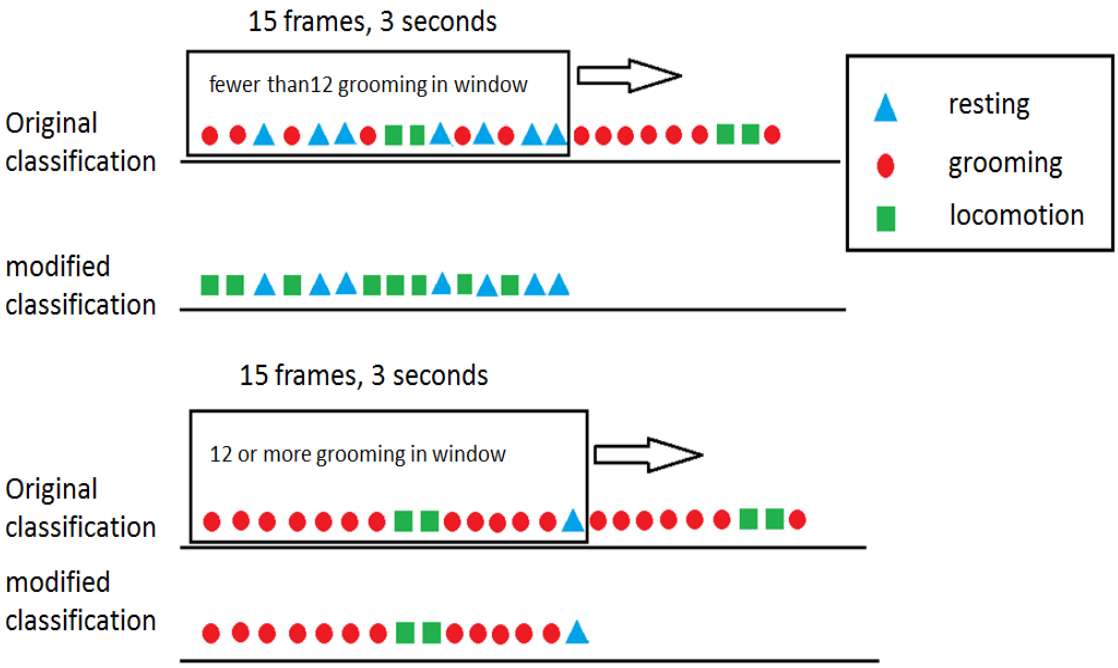

Figure 3.12 Grooming filter scans the output from the classifier and makes changes to some classifications. The objects (red circle, blue triangle and green square) in a row represent the classification of all the frames. The filter will change some grooming frames to locomotion frames if they don't meet the criteria.

To minimize this interference, a grooming filter is applied to work on the array of identified results (Figure 3.6). The filter is a window proceeding from the first frame to the last which sequentially examines the frames. For each step, the window checks 15 straight frames at the same time. If there are 12 or more frames in the window classified as grooming, these grooming will be finally verified, otherwise these "grooming" frames will be marked as "temporary". When the window moves to the next step, one more frame is loaded in the window and one will be moved out, so the number of "grooming" frames in the window may be different from last step and those "temporal" grooming frames on the left in the window from last step have a chance to be verified and get rid of the "temporal" mark. The grooming frame will not be marked as "temporal" once being

verified. After the filter finishes all the work from the first frame to the last, those frames which are still marked as “temporal” will be reclassified as locomotion because they failed to meet the criteria of grooming.

3.7 Error analysis

The error of grooming identification is reflected in two aspects and I am illustrating in detail with Figure 3.13. The outer rectangle frame represents all behaviors in a video. The red circle represents all the behaviors identified as grooming by the method, and the green circle represents all the behaviors that are actual grooming. The ideal result is that the two circles completely coincide, since the overlapping area of the two circles (area 1) represents grooming detected correctly by program. When the two circles do not coincide, there are two non-overlapping areas, representing two different types of error. The non-overlapping area of the green circle (area 3) represents behaviors that are grooming but are not identified as grooming by the algorithm. The non-overlapped area that belongs to the red circle (area 2) represents behaviors that are not grooming but are mistakenly identified as grooming by the algorithm.

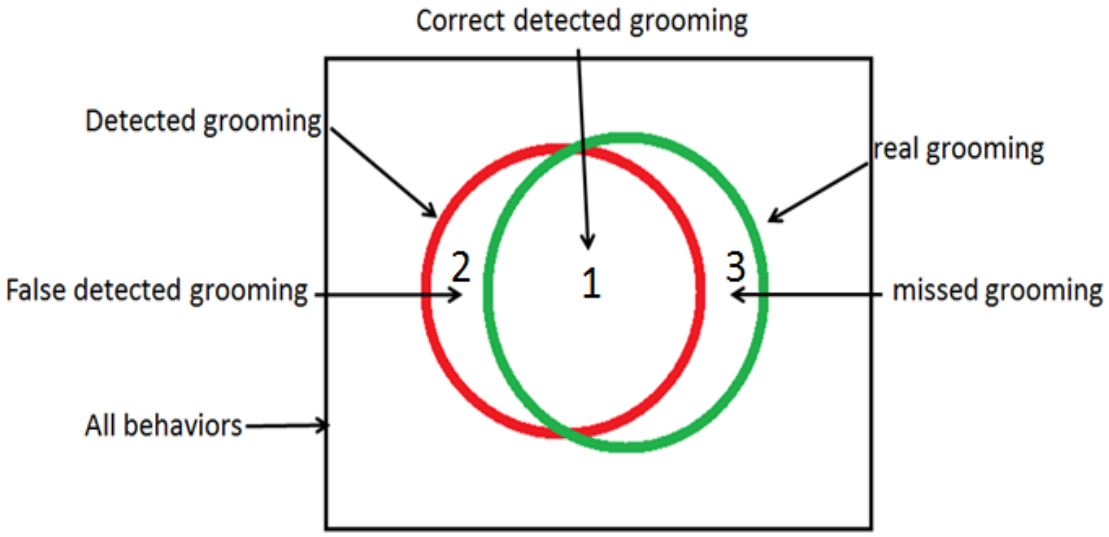

Figure 3.13 A cartoon shows the relationship between real grooming and program-detected grooming. In every frame the fly's behavior must be either grooming or not grooming. The area inside the green circle represents those frames in which the fly is indeed grooming (confirmed by eye). The area inside the red circle represents those frames in which the fly's behavior is identified as grooming by the program. When two circles do not coincide, it is because the program falsely identified some other behaviors as grooming (represent as area 2) and neglected some real grooming events (represent as area 3).

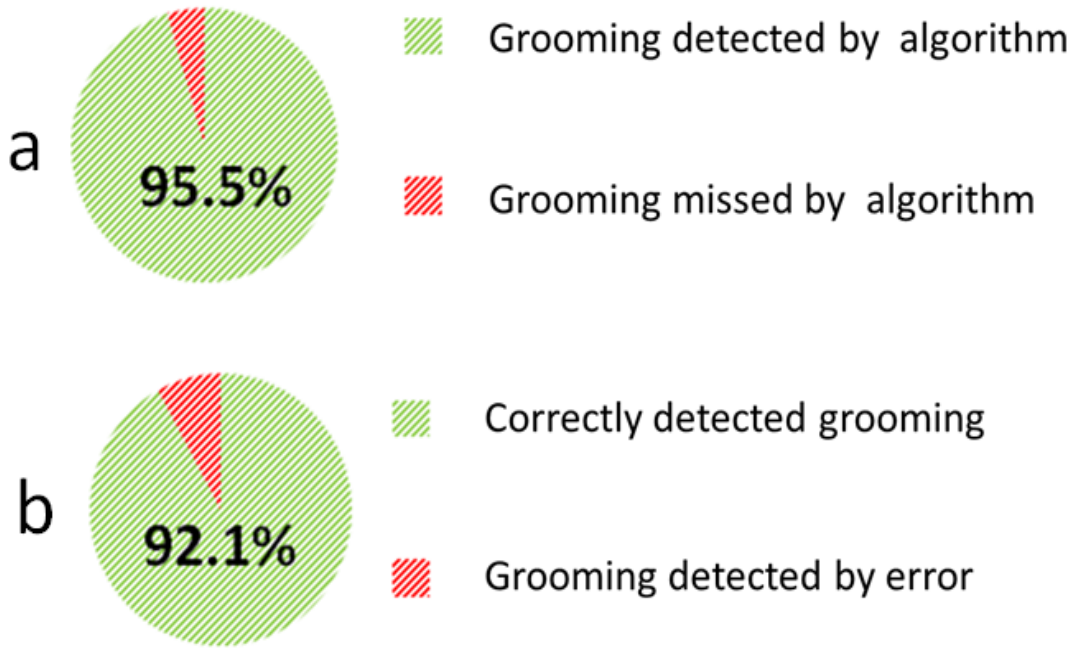

Figure 3.14 Estimates of method error in two aspects
For the two aspects of error, we estimate them in two ways. Chart (a) shows the ratio of overestimated grooming, which is the ratio of falsely detected grooming to detected grooming in total. Chart (b) shows the ratio of underestimated grooming, which is the ratio of neglected real grooming to all the real grooming. The data we used for error analysis is an eight-hour video of 2U wild type flies (n=20).

As displayed in the pie chart above, program detected 95.5% of all grooming events in the video.

In order to estimate error in our method, we randomly sampled 20 time intervals, 20 or 30 minutes each. Then we manually found the starting and ending time of all grooming of randomly chosen flies from all 20 flies during those periods of time. We manually added up the total grooming time of every fly in those intervals. Then we calculated the total time of grooming detected by the program in those intervals. We sampled 460 minutes. According to the manually checked results, the total grooming time in the video takes up

to 2107 seconds among which 2012.2 seconds were accurately classified as grooming by the algorithm. This indicated that our program successfully detected 95.5% of true grooming in the sample (Figure 3.14a). According to this video, about 45% of the neglection of grooming by the algorithm happened when the flies were shifting position while grooming. Another 35% neglections attribute to fly movements being significant larger than typical grooming movement on average.

92.1% of the grooming identified by the program was correct.

In order to check the rate of false positives, we randomly sampled 10% of all grooming found by our program, and then manually checked the false positive result by closely examining the video. The total duration of the grooming sample was 5018 seconds, and the false positive time was 396 seconds (Figure 3.14 b). We also found that low-speed movement (centroid moves slowly) caused 82% of the overestimated results, and, the combination of bending of the abdomen and stretching and contracting of the mouth counted for 12% of the overestimation. Other possible reasons also include leg stretching and background noise.

CHAPTER 4

Daily Activity of Grooming

With the help of the tracking system we are able to identify flies' grooming events over long times. Grooming and locomotion are similar in that they both include limb movement, but they obviously have different function. This chapter puts grooming and locomotion into comparisons to reveal their relationship.

4.1 Grooming varies less during day and night compared to locomotion

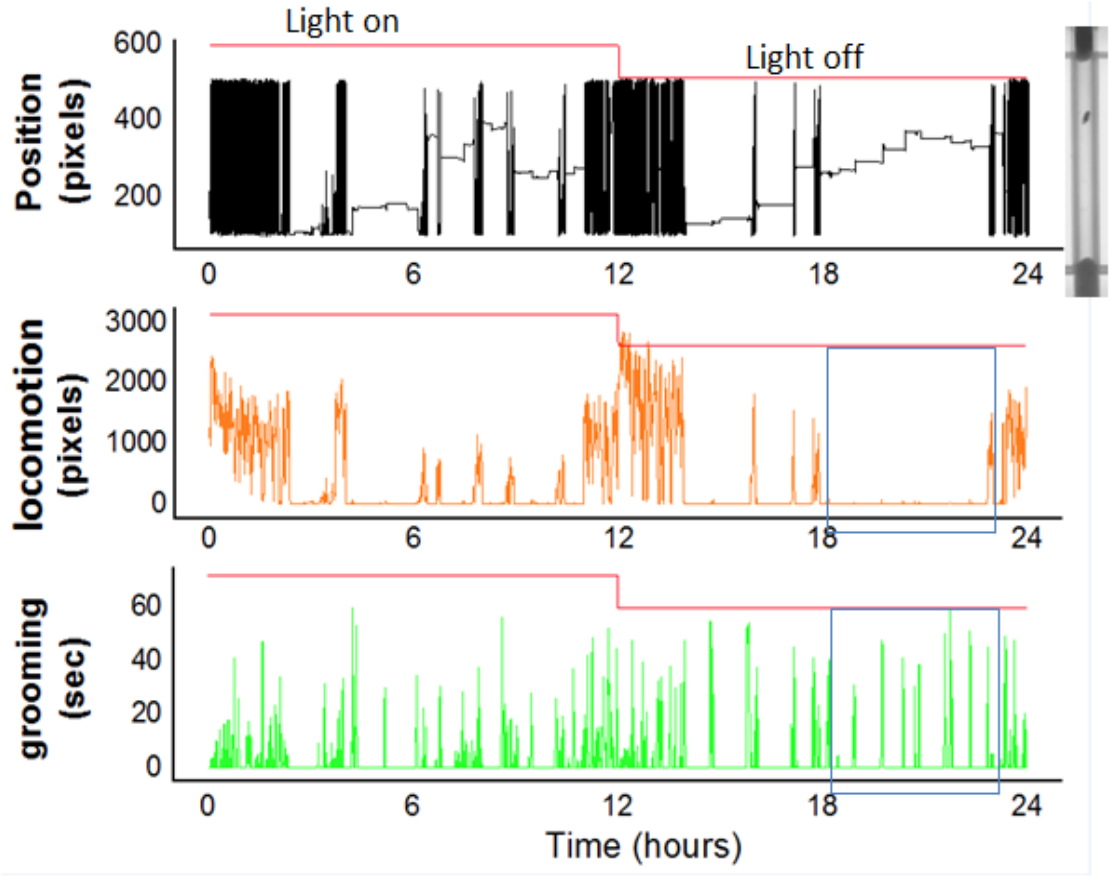

Figure 4.1 Position (top row), locomotion level (middle) and grooming level (bottom) of a single fly during two days in LD cycle followed by two days in DD cycle. Locomotion level shows the displacement (× 100 body lengths) in every minute bin. Grooming level shows the total grooming time (second) in every minute bin. White/black bars indicate light/dark, respectively.

We compared one days' grooming level (time of all grooming events in every minute) of one fly with its locomotion level (displacement in every minute) to see if these two behaviors share any similarity in fitting into daily routine (Figure 4.1). The locomotion activity has been studied by *Drosophila* Activity Monitoring System (DAMS) and video tracking before, and our locomotion activity data are consistent with the previous studies [16]. As expected, locomotion level shows bursts around the time when light being turned on and off in light-dark cycle (LD), which are known as morning and evening peaks, respectively. Similar to locomotion, grooming events also occur more frequently around light transition, but not as drastically as locomotion changes over a day. In the middle of day and night locomotion drops dramatically and happens sporadically. In contrast, grooming still keeps on a considerable level and frequently occurs.

Comparing with locomotion, grooming is more frequently and constantly required. Flies groom quite often. The data show that the number of grooming events of one day varies from 500 to 1500, which means that fly grooms 20 to 60 times per hour on average. This frequency increases around the time point of light transition. As we can see from Figure 4.1, in the middle of day and night, some intervals between two grooming are tens of minutes or even longer. Meanwhile the locomotion activity also stays at low level. But even when the locomotion level is close to zero for several hours, indicating that the fly is in the most inactive state, the longest interval between two grooming events is still within one hour (Figure 4.1, blue rectangle). The constant need for grooming suggests that maintenance of body surface, i.e., grooming once per hour is necessary for flies.

4.2 **Daily grooming amount is uniform among individuals and is not significantly influenced by locomotion amount**

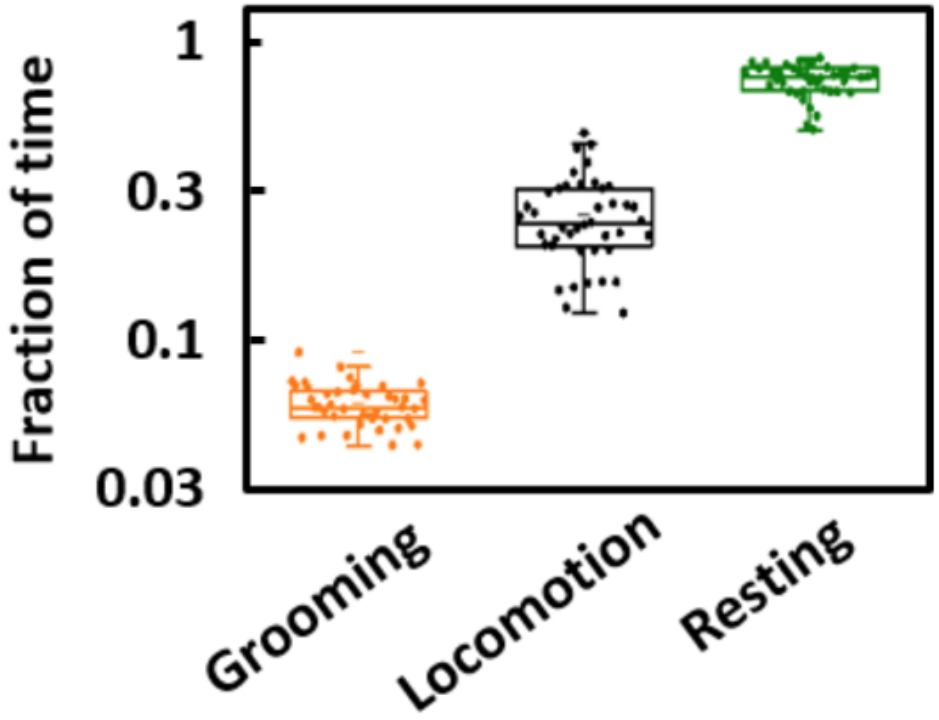

Figure 4.2 Fraction of time spent on grooming, locomotion and resting states. On average, flies spent about 6% of time grooming every day and 30% of time on locomotion (N=42 Canton S flies).

The time spent on grooming is about 6% of all the time, while time spent in locomotion is 30% (Figure 2C). Despite the time spent on grooming everyday being shorter than locomotion, it occurs more consistently among individuals than locomotion. We calculated all individuals' grooming amount per day and divided it by population mean (Figure 4.3), and find that the daily grooming amount of different individuals are significantly more concentrated around the population mean than locomotion, indicating that the time spent on grooming per day is relatively uniform and is not strongly affected by the activity intensity of the fly.

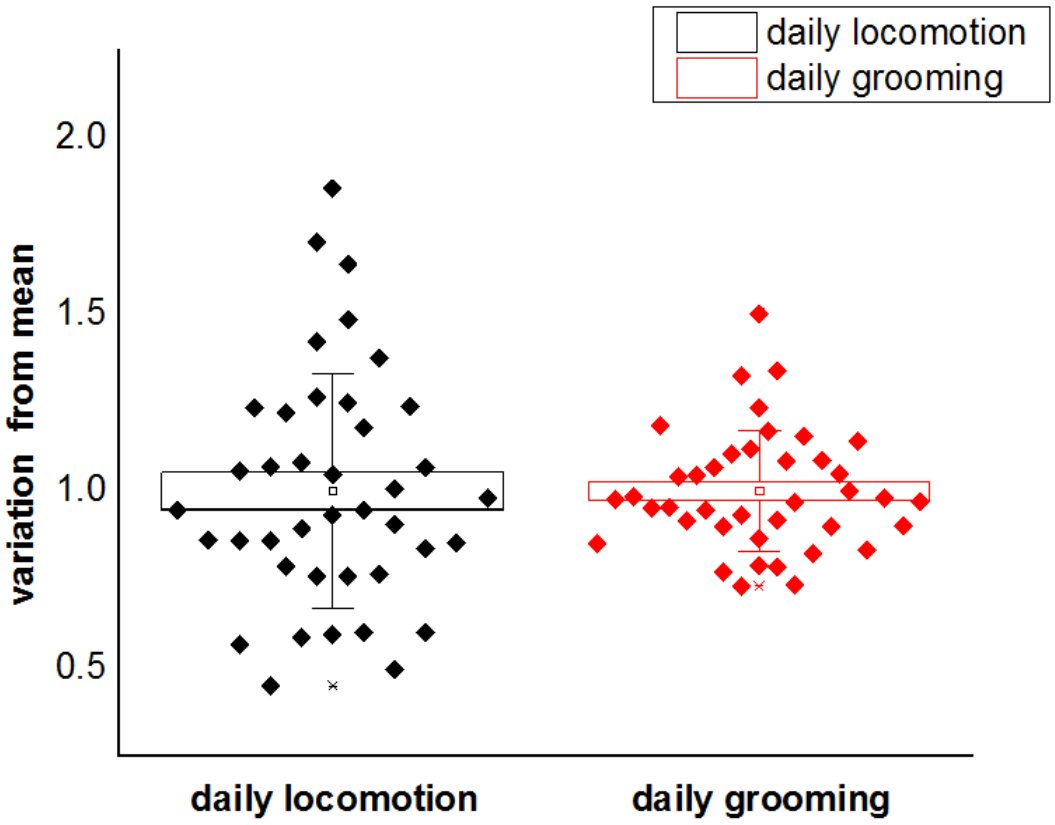

Figure 4.3 Individual differences in daily grooming and locomotion. Black and red points represent daily grooming and locomotion times, respectively, compared with population mean (daily activity amount of individual divided by mean). Under both LD and DD conditions, variation in daily grooming time among different individuals is obviously less than the variation in locomotion (In LD, the value of standard deviation of grooming is 0.164, equals less than half the value of locomotion, 0.329 In DD, the two values are 0.147<0.27, N=40 Canton S flies).

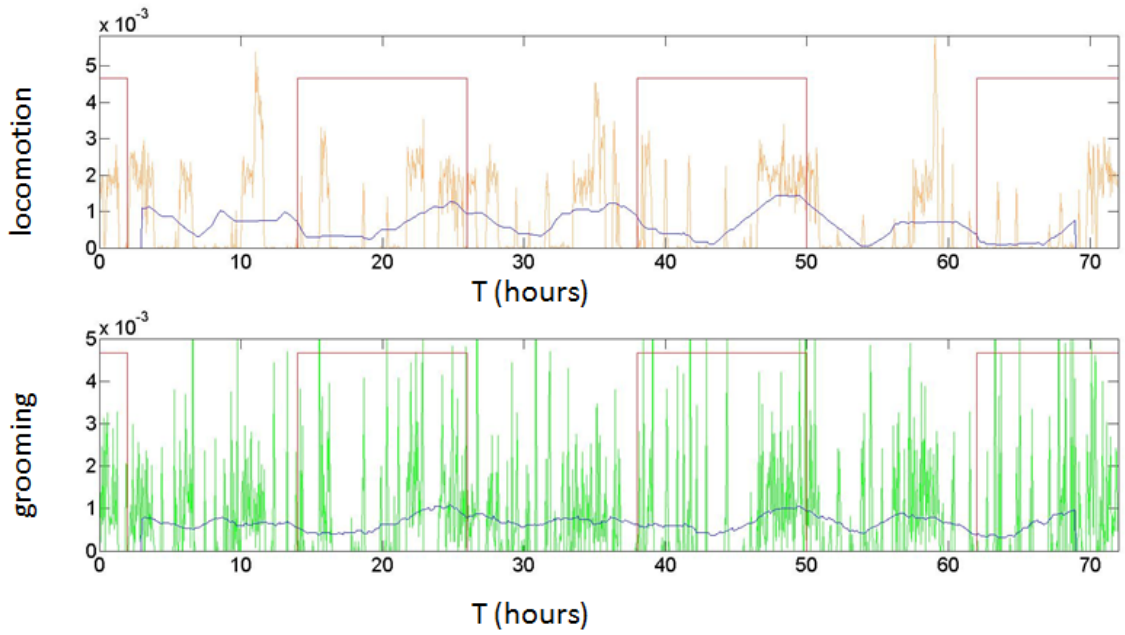

Figure 4.4 The trend of grooming and locomotion variation for three days. Each point on the blue lines shows the average value of grooming and locomotion in a three hour window.

To check the correlation of grooming and locomotion in a larger time scale, I plotted the variation trend of the two behaviors for three days (Figure 4.5). The crests and troughs of grooming and locomotion generally occurred at the same time, but the oscillation amplitude of grooming is smaller than that of locomotion. Since we already know that the clock neurons are responsible for the pattern of locomotion, it is possible that the relative weaker rhythm of grooming is caused by the rhythm of locomotion.

To test how grooming relies on locomotion, we recorded flies in LD cycle and DD cycle. Since the lack of light could cause a significant reduction in locomotion, it should lead to a reduction in grooming if grooming is directly influenced by the level of locomotion.

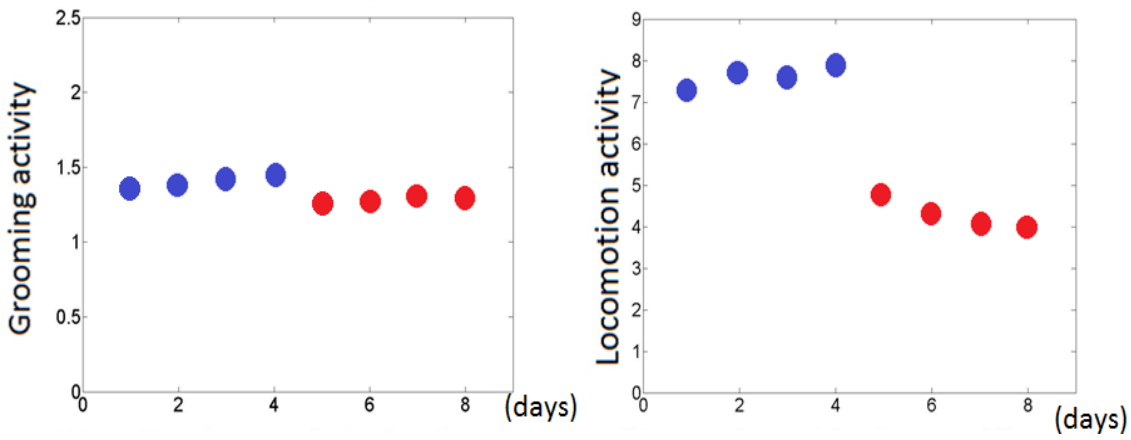

Figure 4.5 Grooming and locomotion level in LD and DD cycle. Flies are being recorded in LD cycle for four days followed by four days in DD cycle. The level of grooming amount decreased only 9%, while the level of locomotion decrease 47%. Grooming and Locomotion activities are in arbitrary unit.

We monitored the flies for four days in LD and then another four days in DD. As expected, the locomotion is strongly affected by lighting condition that the total displacement of fly per day decreases to 53% of the level in LD. Meanwhile the grooming level merely decreased 9%. The relative change of grooming is much less than the relative change of locomotion. This implies that the locomotion level does not seem to effect grooming level directly.

CHAPTER 5

The Circadian Rhythm of Grooming

This chapter explains the circadian rhythm of grooming and the compares grooming, locomotion and feeding rhythm.

5.1 Grooming performs a circadian rhythm that resembles locomotion's

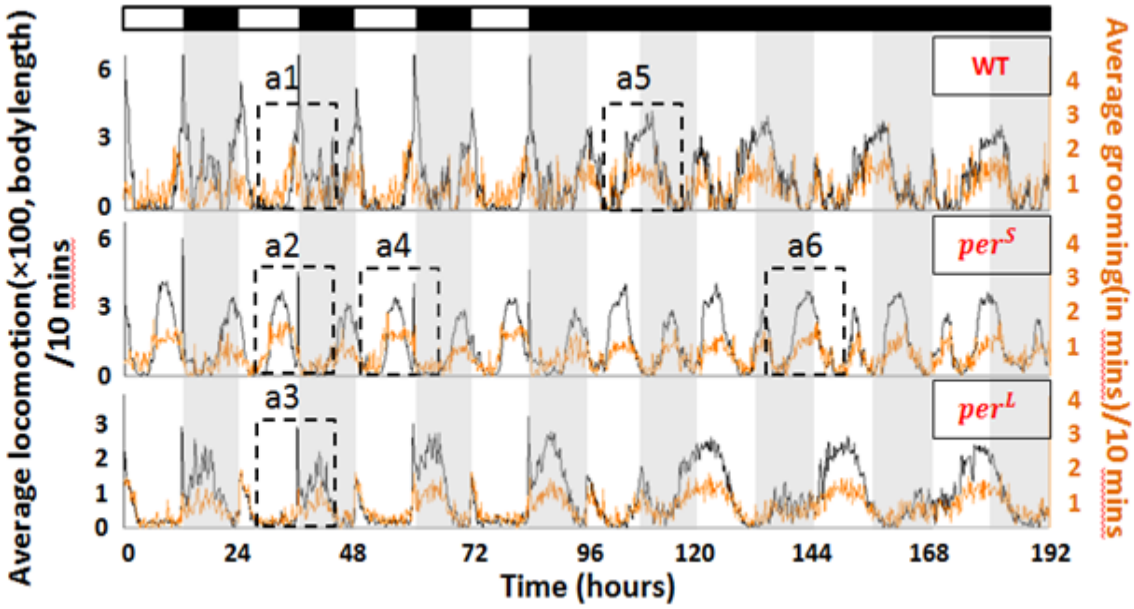

Figure 5.1 Grooming rhythm under LD and DD light condition. Locomotion and grooming activity (in 10 minute bins) of wildtype and clock mutants during four days in LD cycle followed by four days in DD cycle. Both activities are population averages (N=10 wildtype, 8 per^S, 10 per^L). In DD, wildtype flies continue to show 24hr rhythms in grooming and locomotion. Mutations not only affect locomotion rhythms but also rhythms of grooming. In all three populations, grooming and locomotion are generally in phase. Dashed rectangles expanded in Figure 5.2. The correlation coefficients between grooming and locomotion are 0.44, 0.46 and 0.59 in wildtype, $per^S$and per^L, respectively.

The regular morning and evening peaks of grooming are shown in both LD and DD cycle, suggesting that the modulation of grooming may have a stable rhythm. To test whether grooming has circadian rhythm or not, we monitored Canton S, per^S, per^L and per^0 flies four days in LD and four days In DD. In LD cycle, all types show 24-hour grooming rhythms. However, these rhythms perform differently on the occurrences of morning and evening (M/E) peaks. For example, the differences of evening peaks are highlighted in Figure 5.2, frame a1-a3: for Wildtype fly, the evening peaks are very close to the time of light transition; for per^S fly, the evening peaks are about two hours in advance to light transition; for per^L fly, the evening peaks are about three hours later than light transition. We can conclude that the differences in internal clock rhythms cause the grooming peaks shift in LD cycle, indicating that the morning and evening peaks are not just a response to light condition. The early peaks in per^S and late peaks in per^L suggest that the expectation of light transition is clearly related to circadian clock. In DD cycle, grooming rhythms are consistent with their clock's periods: per^S shows shorter cycle, per^L shows longer cycle and wildtype keeps 24 hour cycle. The periods of grooming and locomotion in DD are shown in Figure3 B. Grooming's period is exactly the same with locomotion's periods for the same clock type. In addition, since the morning and evening peaks of grooming and locomotion appear at about the same time, the variations of grooming and locomotion are generally in phase. The correlation coefficients between grooming and locomotion are 0.44, 0.46 and 0.59 in wildtype, $per^S$and per^L, respectively, showing that grooming are medium correlated to locomotion.

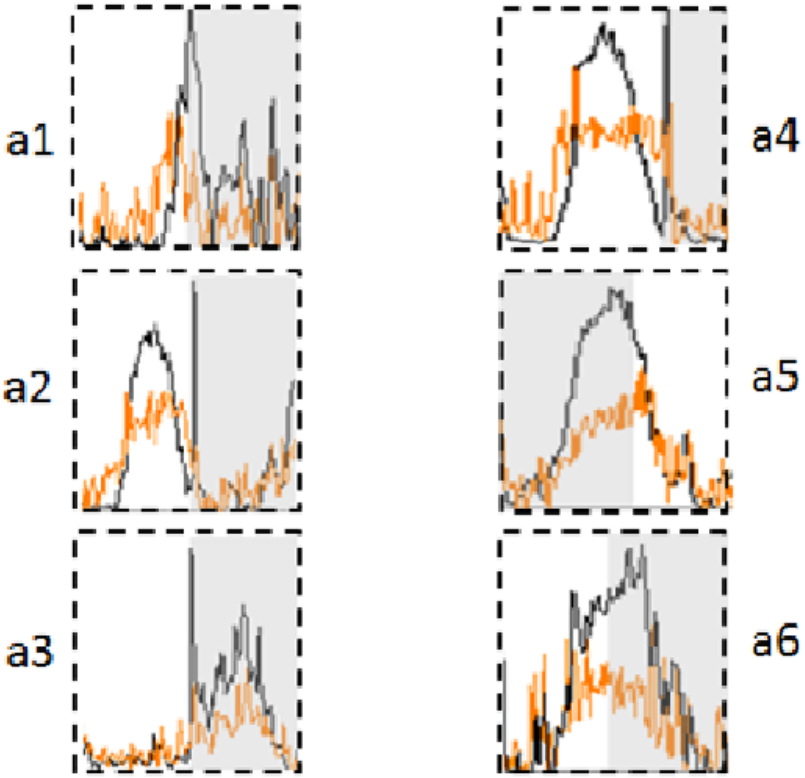

Figure 5.2 a1-a3 show the grooming evening peaks are affected by the clock gene. Short period gene causes an anticipated increase earlier than wild type's, while Long period gene cause a delay. a4-a6 show the details of morning and evening peaks, suggesting that the variation in grooming is not positively correlated to locomotion.

Although these concordant changes in grooming and locomotion once again suggest that the grooming movements may be a subset of the more robust locomotion activities, close inspection of temporal patterns in the data indicate that grooming is independently controlled by the clock. In Figure 5.2, frames a4-a6 highlight details of grooming and locomotion's variation around peak, clearly showing that locomotion and grooming peaks have different shapes and their activity levels could vary in opposite directions.

This indicates that changing of grooming's rhythm may instead be due to independent regulation and not a response to locomotion.

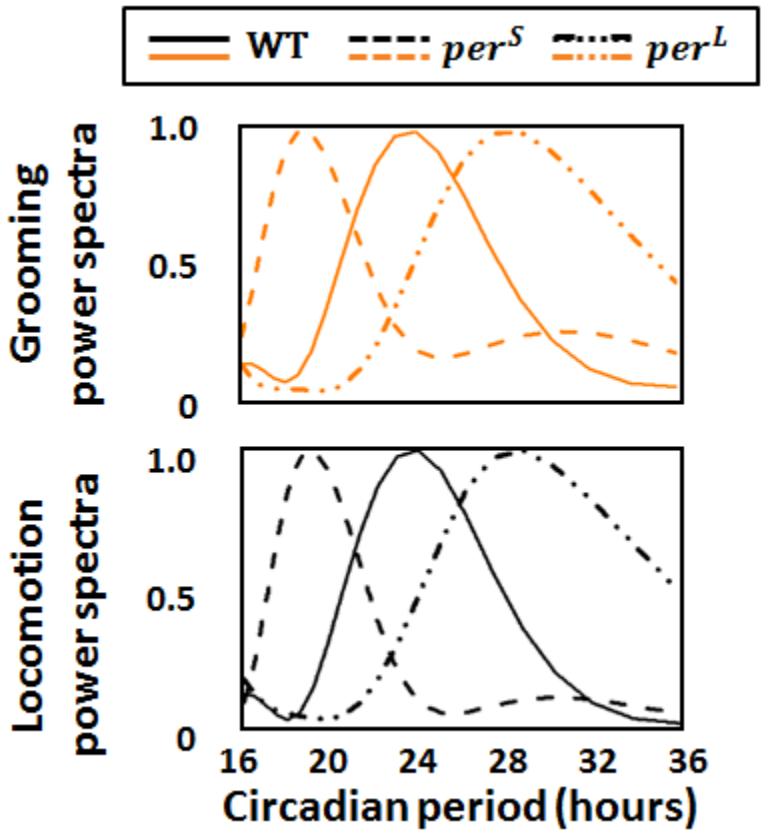

Figure 5.3 Power spectra of grooming and locomotion. Average power spectra of grooming and locomotion of wildtype, per^S and per^L flies in DD (N=48 wildtype, 8 per^S, 10 per^L). Wildtype controls show period of 24 hours, per^S 19 hours, per^L 29 hours.

5.2 Grooming's circadian rhythm does not resemble to feeding's

The method to identify feeding activity:

Since our recording system only provides image data, we cannot measure how much food the fly consumes, but we can evaluate feeding activity from the video. The program counts the time a fly spends with its centroid close enough to make a contact with food. If

the fly keeps stationary for more than 5 minutes the fly is considered to be resting so even if the fly is close to food, it will not be considered to be feeding. Only when the fly is staying on the food while being active do we consider it to be feeding. We analyzed feeding activity for four days and compared it with grooming and locomotion (Figure 5.5)

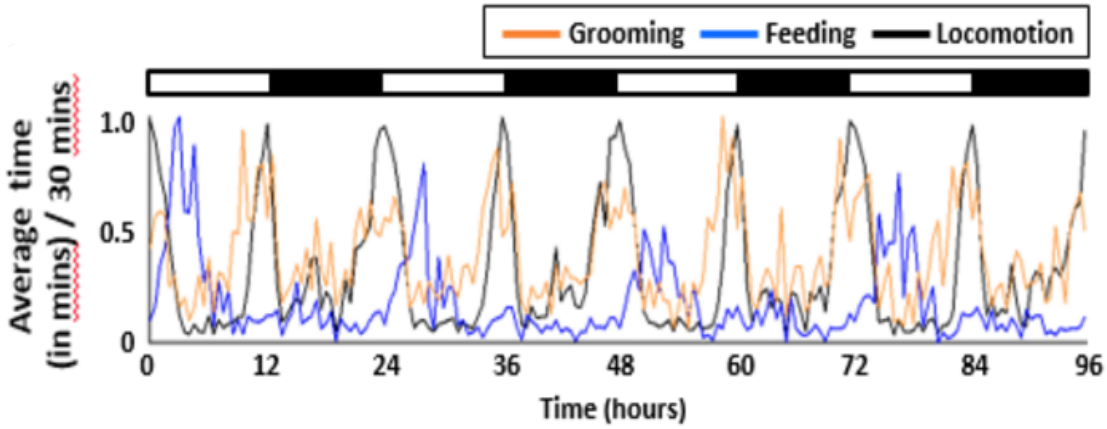

Figure 5.4 Normalized average (n=6) level of grooming (orange), feeding (blue) and locomotion (black) during 4 days in LD. Bursts of feeding happen 3-5 hours after the morning peak of locomotion. The correlation coefficient between grooming and feeding is -0.25, indicating they are slightly anti-correlated.

For feeding behavior, there is only one activity peak each day, and it occurs clearly at a different time from grooming – about two or three hours later than morning grooming peak (Figure 5.4). There is no significant change in grooming level around feeding's peak, indicating that feeding is not closely linked to grooming's regulation, at least not as closely as locomotion does. Analysis shows that grooming and feeding are slightly anti-correlated, which also indicating that the regulation of grooming is not affected by feeding. However, we can see that the evening peak of grooming (about seven or eight hours after the feeding peak) always occurs a little earlier than locomotion's evening

peak. If this early appearance is related to feeding, it means that feeding does not bring the immediate drive to groom, but affects the grooming level several hours later.

5.3 Head grooming and body grooming perform different circadian rhythms

Fly grooming consists a series of actions that engages different part of body. People have found that when fly needs to clean its whole body, it uses different types of grooming action in a certain sequence. The grooming has a certain order as follows: starting from eyes, and then antenna, to head abdomen, wings and thorax at last [24]. Apparently cleaning the sensory organs on the head has higher priority than cleaning the body.

It remains unknown if the circadian clock regulates different types of grooming action together or separately. Is the level of different types of grooming action always proportional to the total grooming level, or they have their own schedules? To test it we classified all the grooming events detected into 'head' grooming and 'body' grooming by the size of movement (CM+CP). 'Head' grooming has relatively smaller range in movement than body grooming. Head grooming roughly includes the movements of front legs, antennae and eyes, while' body' grooming has relatively wider range in movement. It roughly includes all other grooming events. According to the grooming action sequence mentioned in previous paragraph, the head grooming has higher priority than body grooming. We compared the schedules of the two grooming types (Figure 5.5) and found that they are alike in general. Both are quite close to the trend of total grooming (the rhythm shown in Figure 5.1). The correlation coefficients between body grooming and head grooming are 0.93, 0.88 and 0.96 in wildtype, per^S and per^L, respectively.

Despite the two types of grooming are highly-correlated, they deviate from each other near day night transitions (example in dotted frames in Figure 5.5). The deviation appears even more obvious in DD, indicating that the deviation is not because of the present of light cycle but the internal regulation. The different schedules of head and body grooming suggest that different types of grooming may be controlled separately by the circadian clock. But the high correlation coefficients indicate that different types of grooming may share part of regulation system.

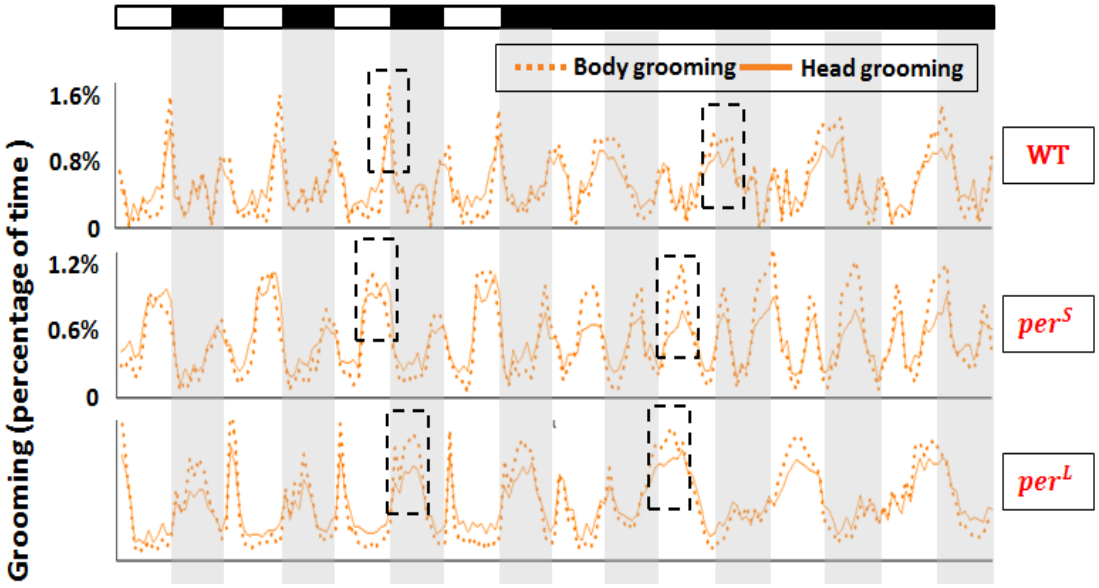

Figure 5.5 The different schedules of head and body grooming. Head grooming includes the movement of front legs, antennae and eyes, while body grooming includes all other grooming events. The lines show each type of grooming time in 10 minute bins and normalized by total grooming time of respective type (HG/TG. HG: time spent in grooming per hour. TG: total time in grooming). The two schedules are to a large extent similar but deviate from each other around day night transition times (see dashed rectangles). The correlation coefficients between body grooming and head grooming is 0.93, 0.88 and 0.96 in wildtype, per^S and per^L, respectively.

CHAPTER 6

Discussion

This work provides an approach which for the first time acquires the long-term data needed to show the modulation of grooming of *Drosophila*. My research reveals that grooming has a circadian rhythm and has a variation trend similar to locomotion. These findings allow us to study the control of grooming from a new perspective.

Previous studies proved that grooming events could be triggered by external stimuli such as dust and chemical irritants. However, these stimuli may not be responsible for many grooming events since grooming could happen without any apparent external stimuli. Hygienic requirement would be a good explanation for the non-stimuli-triggered grooming. However, it still remains unclear how the fly's body surface gets dirty and how these non-stimuli-triggered grooming are controlled. If a fly is coated with dirt, it would groom immediately. But particles which are too small are unlikely to trigger a grooming event. An animal in nature will inevitably be adhered to by small particles and parasites over time, and the animal has to remove those particles eventually. But the question is, with what strategy or mechanism does an animal determine when to start a grooming event?

One hypothesis is that a grooming event will be triggered when the amount of particles accumulated on the surface reaches a threshold. And the possible reasons for the surface gradually getting dirty are feeding and locomotion: when the fly is feeding it contacts the food and this may cause the food debris to adhere to fly; locomotion may

cause contact to small particles in the environment. An inference of this hypothesis is that the rhythm of grooming should relate to the rhythm of locomotion and feeding.

The schedule of feeding only has one peak every day and this peak appears about two hours after grooming's morning peak. The result shows that feeding doesn't seem to relate to grooming's schedule so much as locomotion does, since feeding's schedule is much less similar to grooming's than to locomotion's.

It turns out that the rhythm of grooming resembles the rhythm of locomotion, and this result seems to be consistent with the inference of the hypothesis. However, close inspection of temporal patterns shows that grooming sometimes doesn't change in the same direction as locomotion does, especially around the activity peaks. If grooming and locomotion do not change in the same ratio, it could be explained that the cleaning time and cleaning effect are not linearly related. But if they change in different directions so obviously, then it has a conflict with the previous hypothesis. Also, the grooming during static time also supports the idea that grooming is not a passive response to locomotion.

The details of the schedules indicate that, despite the active time of these two different behaviors overlapping, the control of grooming and locomotion are separate. The circadian rhythms of different types of behaviors are also separate. We classified the grooming events into head grooming and body grooming and find out that these two types of grooming have similar schedules, but they still have significant difference.

I speculate that the fly actively regulates grooming events depending on its potential need for surface sanitation. During the time when locomotion level is high, the fly will devote more time to grooming to fulfill the potentially increasing hygienic need caused

by more intensive locomotion. Since grooming is not directly caused by locomotion, they differ in detail, which is supported by our experiment result.

Beside the circadian clock, we are also applying the tracking-method to explore more internal factors that affect the grooming control. For example, bacterial infection may also affect grooming's active regulation and lead to a change of the overall amount of grooming in a day. Infection can reduce grooming level, so lack of grooming is an important behavioral sign for many mammals that are sick with an infectious disease [64]. For example, animals which have been sick for several days or longer often have a scruffy-looking coat. The scruffiness noted in many species, including rodents, felids and ungulates, is likely due to a marked reduction in grooming. Now we are studying the details of the relationship between grooming and disease in *Drosophila*, especially how grooming is changed under different stages of disease. This study may reveal a relationship between grooming and the immune system that is much closer and deeper than we ever thought.

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