

# Studies on the Behavioral Effects of Dopamine in Honey Bees

(*Apis mellifera*)

(ドーパミンがミツバチの行動に与える影響に関する研究)

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## List of abbreviations

### Abbreviation

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DA	Dopamine
OA	Octopamine
D1	Dopamine receptor D1 family
D2	Dopamine receptor D2 family
AmDOP 1	Apis mellifera dopamine 1 receptor
AmDOP 2	Apis mellifera dopamine 2 receptor
AmDOP 3	Apis mellifera dopamine 3 receptor
cAMP	Cyclic adenosine monophosphate
AmOA 1	Apis mellifera octopamine 1 receptor
M	Molar
GLM	Generalized liner model
LRT	Likelihood ratio test
Flupen	Dopamine antagonist flupenthixol
H	High
L	Low
S	Saline
μl	Microliter
GLMM	Generalized liner mixed model
d.f	Degree of freedom
m	Meter
s	Second
g	Gram
JH	Juvenile hormone

## General introduction

Behaviors in social insects are plastic and change responding to the environment. The behavioral changes are controlled by neural factors. The nervous system in social insects is divided into two highly structured, intertwined systems. The first is the visceral (also called stomatogastric or sympathetic) system that controls alimentary canal movements and is closely concerned with the process of neurosecretion. The second is the central nervous system, which coordinates the peripheral sense organs and muscles. Brain is a large group of neurons that lies above the esophagus. For that reason it is sometimes called the supraesophageal ganglion. Three parts are generally recognized. The most anterior section, called the protocerebrum, is the most complex part of the insect brain. It directs neural traffic at the crossroads between sensory input and motor output. At each side, optic lobes extend to the compound eyes; at its center, a pair of large mushroom bodies process olfactory information and control tasks that require visual coordination of locomotor activity and spatial orientation. The mushroom bodies and associated cells provide a structure for elaborate interconnections that allow learning and memory to occur. The second brain section, called the deutocerebrum, connects to the antennae. Its neurons are of two types—one type processes chemosensory information; the other, mechanosensory. The third and smallest part of the brain, the tritocerebrum, connects the central nervous system to the ventral nerve cord through the circumesophageal connectives. It also innervates the labrum (upper lip), pharynx (region between mouth and digestive system), and the rest of the visceral nervous system. The nervous system in social insects with fewer numbers of neurons compared to vertebrates regulates the behavioral changes adequately. The studies of neurophysiological mechanisms controlling the changes of behaviors are important to understand the functions of nervous system in social insects.

Biogenic amines are neurophysiological factors that act as neurotransmitters, neurohormones and neuromodulators and regulate behavior and physiology in animals (Blenau and Baumann, 2001; Evans, 1980; Carlson, 2013). The biogenic amines include catecholamines (dopamine, norepinephrine and epinephrine), phenolamines (tyramine and octopamine), indolamine (serotonin) and histamine. Some of the biogenic amines, including dopamine (DA), serotonin, and histamine, are commonly found in the central nervous systems of both vertebrates and invertebrates, whereas others, including octopamine (OA) and tyramine, are primarily found in invertebrates (Evans, 1980, 1986; Blenau and Baumann, 2001, 2003; Roeder, 2005; Roeder *et al.*, 2003). They involve in the regulation of various behaviors e.g. feeding behavior (Long and Murdock, 1983; Lent, 1985), aggression (Summers and Greenberg, 1995; Huber *et al.*, 1997), sexual behavior (Linn and Roelofs, 1986), locomotor activity (Draper *et al.*, 2007; Lima and Miesenbock, 2005), flight (Sasaki and Nagao, 2013; Claassen and Kammer, 1986; Buhl *et al.*, 2008 ), and learning and memory (Hasselmo, 1995; Harvey, 1996) in different kinds of animals by acting on peripheral and central nerve system.

Honey bees are highly social insects and their various behavioral performances attract the attentions. A group of thousands of kinship bees are called colony, make a society of honey bees. A colony of honey bee is consisting of queens (reproductive female), drones (male) and workers (infertile female), every member characterized by specific behavioral performances take a part in a honey bee society. The behavioral performances of a queen could be divided into two parts: first, when a queen is virgin she fights with a sister-queen for survival, destroys the sister-queen cells that still not emerged and takes mating flights. Second, after mating her ovaries develops and starts egg-laying that she can lay thousands of eggs per day, and her mandibular glands develops that she releases her strong pheromones, chemical signals to the workers which control

many of their behaviors. The workers perform more complex and diverse tasks, inside the colony they feed the broods, clean the nest, cap cells, store honey, store pollen, feed and groom the queen, guide other foragers toward the food source by their waggle dance, in front of the nest entrance they perform fanning and defending against their colony opponents, outside the colony they perform foraging to bring honey, pollen, propolis and water to the colony. Drones with large eyes and strong flight muscles are fed by workers and perform mating flight with virgin queens (Winston, 1987; Johnson, 2010). The neurophysiological mechanisms regulating the behavioral performances of honey bees have been the topic of several studies. Biogenic amines as neurophysiological substances are suggested to regulate the behavioral performance in honey bees.

The biogenic amines DA and OA are found in high amount in honey bees and they bind to their specific receptors at surface of target cells to produce the behavioral reactions. Three receptor subtypes of DA have been characterized in honey bees. AmDOP1 and AmDOP2 which are similar to that of vertebrates D1 receptor, and AmDOP3 which is similar to D2 receptor of vertebrates. AmDOP1 and AmDOP2 activation causes increase of intracellular cAMP levels and activation of AmDOP3 causes decrease of cAMP levels (Blenau *et al.*, 1998; Humphries *et al.*, 2003; Mustard *et al.*, 2003, 2005). The OA receptor, AmOA1 also has been found in honey bees and its activation causes the production of cAMP (Grohmann *et al.*, 2003). These biochemical changes taking place in target tissues underlie the physiological and behavioral changes.

The behavioral performance changes associate with the changes of DA levels in the brain in honey bees. In workers the brain DA level increases with age (Harris and Woodring, 1992) and causes the development of ovaries (Sasaki and Nagao, 2001; Dombroski *et al.*, 2003). In addition, affect the age-related division of labor (Wagener-Hulme *et al.*, 1999) and involves in



the regulation of locomotor activity (Beggs *et al.*, 2007; Mustard *et al.*, 2010) but the effect of DA on flight behavior of workers not studied yet. In drones also DA levels in the brain and hemolymph increase with age (Akasaka *et al.*, 2010) and DA regulates the locomotor activity (Akasaka *et al.*, 2010) and flight behavior (Mezawa *et al.*, 2013). Honey bee virgin queens show a high motivation to perform difficult tasks like fighting with sister-queens, destroying sister-queen cells, and mating flights (Gilley and Tarpy, 2005; Pflugfelder and Koeniger, 2003; Butz and Dietz, 1994) under high brain DA level (Harano *et al.*, 2005, 2008), but after mating the brain DA levels decrease and they do not perform these behaviors (Harano *et al.*, 2005, 2008; Winston 1987). It is known that injection of DA agonist enhances the locomotor behavior of virgin queens and injection of DA antagonist suppresses it (Harano *et al.*, 2008). The effects of DA on the other behaviors of virgin queens are not known. Therefore, in chapter 1 and 2, I focused on the investigation of the effect of DA in the regulation of honey bee virgin queens' behaviors.

In chapter 1, I studied the effect of DA on the fighting and aggressive behavior of virgin queens. The fighting is important for survival and becoming the next queen of the colony. Virgin queens fight against each other, and they have grossly high amount of DA in their brain and hemolymph in that period. So, in this chapter I aimed to examine the involvement of DA as neuromodulator in the regulation of the fighting and aggression for survival in honey bee virgin queens. To examine the effect of DA on fighting and aggression of virgin queens I focused on observing the fight winning between sister-virgin queens and the stinging response frequency of DA and DA antagonist injected virgin queens.

In chapter 2, I studied the regulatory role of DA in the regulation of flight behavior of virgin queens. Flight is important for mating because when virgin queens matured they fly to

mate with drones several kilometers away from their hive in the air (Zmarlicki and Morse, 1963). To find the effect of DA on the motivation of flight and flight behavior of virgin queens, I focused on the flight initiation (to find the effect of DA on flight motivation) and flight performance of DA and DA antagonist injected virgin queens.

In honey bee workers, the brain DA and OA levels increase with age (Harris and Woodring, 1992) that associates with behavioral development (Taylor *et al.*, 1992). The effect of DA and OA in age-related division of labor have been reported (Schulz and Robinson, 1999; Wagener-Hulme *et al.*, 1999). The effect of OA on flight attempts has been reported (Fussnecker *et al.*, 2006), but the effect of DA not studied yet. The effect of DA on the locomotor behavior is conserved among queens, drones and workers (Harano *et al.*, 2008; Mustard *et al.*, 2010; Akasaka *et al.*, 2010) and also the effect of DA on flight behavior in queens (chapter 2) and drones is same (chapter 2; Mezawa *et al.*, 2013). Therefore, in chapter 3, I aimed to examine the effect of DA on the of flight behavior in workers and tested the effect of OA, too. Flight is vital for the colony to get enough food for survival and it also contributes to pollination, so very important for Agricultural production. To investigate it, I measured the duration of flight initiation in DA, OA and their antagonists injected pollen foragers.

## **Chapter 1**

# **The influence of dopamine on fighting and aggressive behaviors of honey bee virgin queens**

## 1.1 Abstract

Fighting and aggression are important for self-preservation in animals. Honey bee virgin queens fight against each other for survival. Because the virgin queens have higher levels of DA in the brain with high aggressiveness than do mated queens with low aggressiveness, DA may regulate the fighting and aggression behaviors of virgin queens. Here, I studied the effect of DA on the fighting and stinging response of honey bee virgin queens. I injected two concentrations ( $1.0 \times 10^{-3}$  M and  $1.0 \times 10^{-2}$  M) of DA and the DA receptor blocker flupenthixol into the abdomen of one-day-old virgin queens and observed fighting and stinging responses. DA injection itself did not affect the potential ability of fighting and stinging. Injections of  $1.0 \times 10^{-3}$  M flupenthixol decreased the winning rate significantly, whereas  $1.0 \times 10^{-2}$  M flupenthixol increased the winning rate, indicating the opposite effects on fighting responses depending on the degrees of blockade of DA signalling. In terms of the stinging response,  $1.0 \times 10^{-2}$  M flupenthixol-injected virgin queens stung significantly more often than control and  $1.0 \times 10^{-3}$  M flupenthixol-injected virgin queens. These results suggest an involvement of DA signalling in the regulation of fighting and aggression in virgin queens.

## 1.2 Introduction

Aggression is a common behavioral reaction against opponents in animals. Typically, animals show aggression when they compete for resources, like food and mates, or when they are endangered. The expression of aggression varies among animals and may be regulated by neurophysiological factors (Archer, 1988).

There is only one mated queen in a honey bee colony typically but, during breeding season or when the queen died, workers rear many queen candidates in queen cells. When a new queen emerges, the queen destroys unemerged queen cells and kills other candidates (Gilley and Tarpy, 2005; Harano and Obara, 2004; Winston, 1987). If simultaneously more than one queen emerged, lethal fights occur, and the winning queen becomes the new single laying queen of the colony after mating (Gilley and Tarpy, 2005; Pflugfelder and Koeniger, 2003; Butz and Dietz, 1994). In fighting period, the virgin queens hold each other by using their legs and mandibles to sting (Butz and Dietz, 1994). Stinging is like a shot for hitting the target that acts as a determinative task to win the fight; the queen that successfully hits the target (inject the poison) first is the winner (Gilley, 2001). Some factors, like age (Schneider and Hoffman, 2008; Tarpy *et al.*, 2000; Butz and Dietz, 1994), body size (Tarpy and Mayer, 2009) and spraying behavior (Bernasconi *et al.*, 2000) affect the lethal fight in virgin queens. Workers may also have some indirect influence on the fight (Schneider and Hoffman, 2008; Tarpy and Fletcher, 1998; Tarpy *et al.*, 2004) by clumping, grabbing (Gilley, 2001) or vibrating (Schneider *et al.*, 2001), although they do not participate directly by stinging (Butz and Dietz, 1994).

DA as a neurophysiological modulator has been suggested to regulate the fighting and aggressive behaviors in some insects. Two dopaminergic neurons were found to modulate the

aggression in fruit flies and both their activation and inactivation affect fly's aggression (Alekseyenko *et al.*, 2013). DA is necessary for the recovery of aggression after defeat in Crickets (Rillich and Stevenson, 2014). DA stimulates the threatening behavior in *Formica polyctena* workers (Szczuka *et al.*, 2013). DA is also suggested to modulate the aggressive behavior in some mammals as DA receptor antagonists such as haloperidol and sulpiride, and DA presynaptic drugs such as amphetamine affect aggression of human, cats and rats (Patki *et al.*, 2015), despite having different structural organization of the central nervous systems.

In worker honey bees, DA plays role as a key regulator of reproduction. When there is no queen in a colony, brain levels of DA increase in workers, which acts as a stimulator of ovaries to develop (Harris and Woodring, 1992; Sasaki and Nagao, 2001; Dombroski *et al.*, 2003). The relationship between DA levels and reproductive status in queens is completely opposite to that of workers, as their ovaries start to develop after mating (Winston, 1987) while mated queens show decreased levels of DA (Harano *et al.*, 2005, 2008). Compared to workers, queens, especially virgin queens, exhibit DA levels that are several times greater (Brandes *et al.*, 1990; Harano *et al.*, 2008; Harano *et al.*, 2005; Sasaki *et al.*, 2012). These studies suggest that DA has functions other than developing the ovaries in virgin queens.

The main purpose of this chapter was to examine that whether DA modulates the lethal fighting and aggression among young virgin queens or not. To investigate the effect of DA on fighting and aggressive behaviors of virgin queens, I administered DA and a DA receptor blocker, flupenthixol, to virgin queens and observed their fighting and stinging behaviors.

## 1.3 Materials and Methods

### 1.3.1 Queen rearing:

We reared virgin queens by using a standard queen-rearing method (Laidlaw and Page, 1997). Briefly, newly hatched European honey bee (*Apis mellifera*) larvae from queen-right colonies were taken into artificial queen cups using a grafting tool and transferred to queen-less colonies to rear virgin queens in an apiary of Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan. I took sealed queen cells to the laboratory 2 days before expected emergence and kept them in individual plastic containers (diameter  $\times$  height; 135 mm  $\times$  80 mm) at 34°C. One day later, I added 20 nurse bees to the plastic containers and kept them by feeding a mixed solution of 10% royal jelly and 40% sugar until following experiments.

### 1.3.2 Injection of DA and DA receptor antagonist:

To investigate the impact of DA on fighting and stinging behaviors of unmated queens, dopamine hydrochloride (Sigma Aldrich) and flupenthixol dihydrochloride (European pharmacopeia), as a DA receptor antagonist, were diluted by honey bee saline (7.5 g of NaCl, 0.2 g of KCl and 0.2 g of  $\text{CaCl}_2 \text{ L}^{-1}$ , pH=6.7) to  $1.0 \times 10^{-3} \text{ M}$  or  $1.0 \times 10^{-2} \text{ M}$ . They were then injected (1  $\mu\text{l}$ /queen) into the abdomen of 1-day-old virgin queens through an intersegmental membrane between the 3rd and 4th abdominal segments using a Hamilton syringe (Hamilton Company) (Mezawa *et al.*, 2013). The control queens were injected with the same amount of saline. They were all anesthetized on ice for 5 minutes for injection. To determine the effect of body weight, the body weights of queens were measured before injection.

### 1.3.3 Observation of fighting behavior:

After drug injections, the virgin queens were paint-marked on the thorax and transferred into a petri dish covered by a piece of red transparent film (to make dark same as field colony condition) (diameter  $\times$  height; 90 mm  $\times$  15 mm). They were kept in the petri dish for 20 minutes before observation to allow for sufficient diffusion of the injected drug within haemolymph. Then, they were moved to an arena, shown in Figure 1-1. The queens were transferred into the cells on each side, and the barriers to the center cell were then opened. I recorded video images of all fights by video camera to know exact timings of initiation and termination of the fights. I regarded that a fight initiated when a queen held the opponent with legs and tried to sting it or they do so each other, and that the fight terminated when a queen left the opponent which was stung and paralyzed. At this moment, I judged that the injured queen lost this fight because they typically dead in a short time. The time to fight initiation from the beginning of trial (opening barriers) and the fight duration (time between the initiation and termination of fight) were recorded for every pair. In natural colonies, sister-queens fight with each other, so we paired sister-queens in the fighting experiments.

In total, 130 (65 pairs) queens were analyzed in fighting experiment. The larvae were grafted from 8 queen-right colonies. I arranged 10 pairs for saline vs saline, 10 pairs for DA low level ( $1.0 \times 10^{-3}$  M) vs saline, 10 pairs for DA high level ( $1.0 \times 10^{-2}$  M) vs saline, 18 pairs for flupenthixol low level ( $1.0 \times 10^{-3}$  M) vs saline and 17 pairs for flupenthixol high level ( $1.0 \times 10^{-2}$  M) vs saline. The queens that liquid come out from their abdomen after injection and the queens that they did not fight were excluded from the analysis, because intact natural virgin queens fight with each other and the chance of both opponents of a pair became zero.



## Fighting experiment

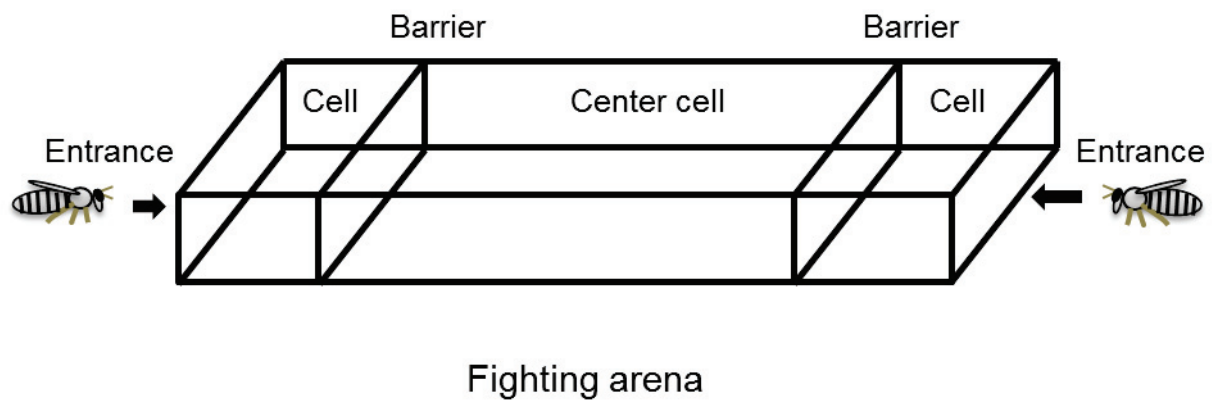
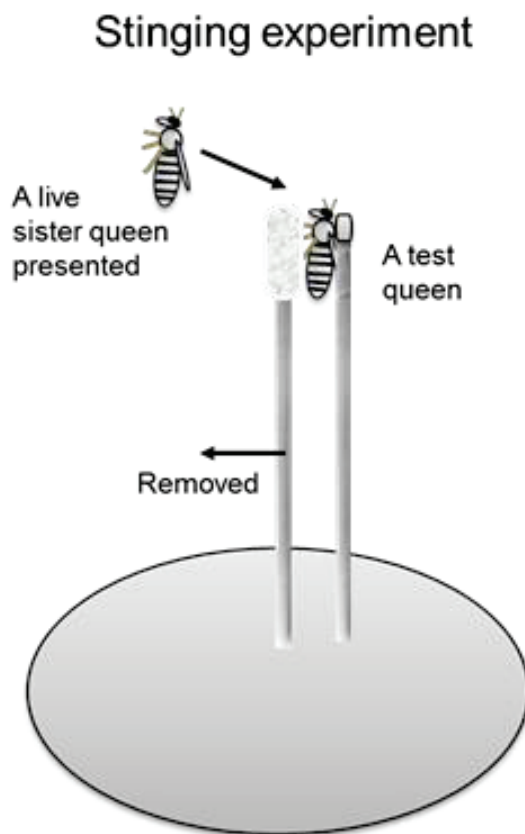


Fig. 1-1. Setups for fighting experiment. Fighting arena. The fighting arena had two entrances, was red covered and was divided into three cells by two red glasses barriers. To observe fighting, queens were inserted into two side cells, after that barriers were removed.

#### 1.3.4 Observation of stinging behavior:

After injection, virgin queens were kept for 20 minutes in a red covered petri dish as described above. Then, I stuck the queen to a toothpick using melted bee wax on the dorsal side of the thorax and their wings according to a method described by Ichikawa and Sasaki (2003). The head and abdomen were free to move. The toothpick was stuck over a sponge vertically. I provided a 10-minute rest and feeding period with another toothpick (having a piece of cotton that was immersed in a 30% sugar solution on the upward side) stuck in front of the queen. Queens could grasp the toothpick with their legs and feed on the solution (Fig. 1-2). To measure the stinging response, I removed the cotton toothpick and presented the dorsal side of a live sister-queen abdomen to the thorax and head of an experimental queen, since a key stimulus that elicits a stinging response to sister-queens is present on the dorsal surface of the queen abdomen (Pflugfelder and Koeniger 2003). Experimental queens were allowed to touch the sister's abdomen with their mandibles, antennae and legs. I counted the number of stinging responses (protrusion of the stinger) for 5 minutes with a counter. During the observation, I did not permit queens to sting each other.

For stinging experiment, 66 queens were analyzed. The larvae were grafted from 4 queen-right colonies. Twenty queens for saline (control), 10 queens for DA low level, 11 queens for DA high level, 12 queens for flupenthixol low level and 13 queens for flupenthixol high level. Same as fighting experiment, I excluded the queens that liquid come out from their abdomen after injection.



**Fig. 1-2. Setups for stinging experiments. The test queen was stuck on a toothpick (height: 55 mm), and a toothpick with 30% sugar solution cotton was placed in front of the queen for resting and feeding.**

### 1.3.5 Data Analysis:

I determined the effect of factors (colony, treatment and body weight) on the result of the fight, the time until fight initiation, fight duration and frequency of stinging of virgin queens by using Generalized linear model (GLM). On the significant effects of factors, I examined statistical tests more detail (colony, treatment and body weight were added as explanatory variables in every model). I applied a binomial distribution for the analysis of the fight result, a gamma distribution for the analysis of the time until fight initiation and fight duration, and a quasi-Poisson distribution (because of over dispersion of the data we did not use a Poisson distribution) for the analysis of frequency of stinging. To consider the significance, I used a likelihood ratio test (LRT).

To evaluate the drugs effect on stinging response, I compared all treatments with control by GLM (LRT) and then followed with Bonferroni p-value adjustment. I performed all statistical tests with R version i386.3.3.3 for windows.

## 1.4 Results

### 1.4.1 The influence of DA and DA receptor antagonist on fighting behavior of virgin queens:

In total, I found a significant effect of treatments on the result of the fight. However, there was no treatments effect on the time until fight initiation and duration of fight (Table 1-1, Table 1-2). There was a significant effect of source colonies on fight duration but not on the result of the fight and the time until fight initiation. The effect of body weight was not significant in all variables (Table 1-1).

DA injection at low and high levels ( $1.0 \times 10^{-3}$  M and  $1.0 \times 10^{-2}$  M) did not affect the results of the fight (binomial tests: low-level DA injected vs. saline injected,  $n = 10$  pairs,  $p = 0.75$ , high-level DA injected vs. saline-injected,  $n = 10$  pairs,  $p = 0.75$ ). Approximately 40% of the drug injected individuals won the fight vs. saline injected (control) virgin queens (Fig. 1-3).

DA receptor antagonist flupenthixol affected the results of the fight. Injection of DA receptor antagonist at a low level ( $1.0 \times 10^{-3}$  M) significantly decreased the winning rate to 17% (binomial test,  $n = 18$  pairs,  $p = 0.007$ , Fig. 1-4), whereas injection of DA receptor antagonist at a high level ( $1.0 \times 10^{-2}$  M) significantly increased the winning rate to 88% ( $n = 17$  pairs,  $p = 0.002$ , Fig. 1-4).

### 1.4.2 The influence of DA and DA receptor antagonist on stinging behavior of virgin queens:

There was a significant effect of treatments on frequency of stinging totally (Table 1-1), but no effect of colonies on the frequency of stinging. Total body weight also did not have significant effect on the frequency of stinging.

The frequency of stinging in flupenthixol high level injected virgin queens was significantly higher than other treatments. However, the frequency of stinging was not significantly different between all other treatments (LRT with Bonferroni  $p$ -value adjustment: Low-level DA injected vs. saline injected,  $n = 30$ ,  $df = 1$ , LR-deviance = 0.002,  $p = 1$ , high-level-DA injected vs. saline injected,  $n = 31$ ,  $df = 1$ , LR-deviance = 3.076,  $p = 1$ , low-level flupenthixol injected vs. saline injected,  $n = 32$ ,  $df = 1$ , LR-deviance = 10.793,  $p = 1$ , high-level flupenthixol injected vs. saline injected,  $n = 33$ ,  $df = 1$ , LR-deviance = 102.4,  $p = 0.00044$ , Fig. 1-5).

**Table 1-1. The effects of colony, treatment and the individual's body weight on the results of the fight, time to fight initiation, fight duration and frequency of stinging.**

<b>Response variables</b>	<b>Explanatory variables</b>	<b>d.f</b>	<b>LR-deviance</b>	<b><i>p</i>-value</b>
Result of the fight	Colony	7	1.163	0.9918
	Treatment	4	22.367	<b>0.0002</b>
	Body weight	1	0.025	0.8734
Time to fight initiation	Colony	7	6.215	0.1502
	Treatment	4	1.340	0.6779
	Body weight	1	0.088	0.6856
Fight duration	Colony	7	13.512	<b>0.0326</b>
	Treatment	4	4.312	0.3003
	Body weight	1	1.364	0.3635
Frequency of stinging	Colony	3	21.579	0.3763
	Treatment	4	141.680	<b>0.0004</b>
	Body weight	1	18.707	0.1011

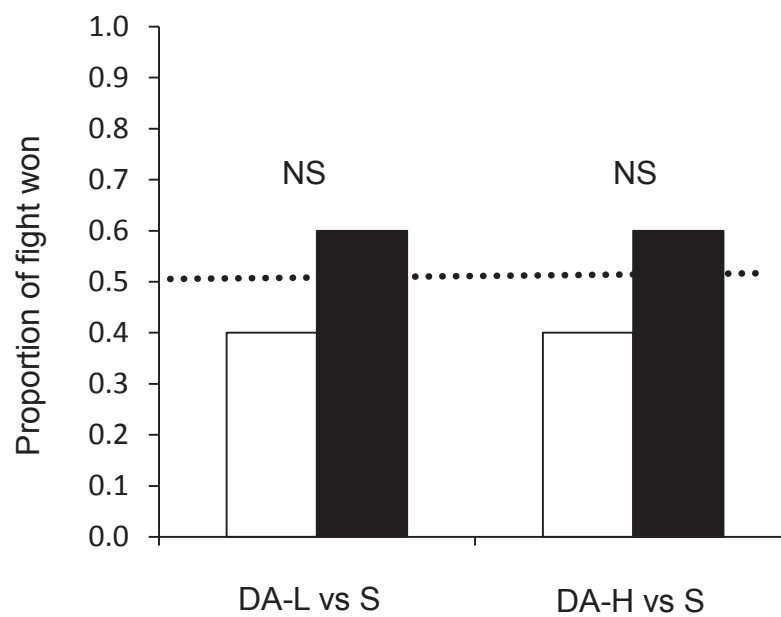
d.f : Degree of freedom

**Table 1-2. Fight initiation and duration time (seconds) in virgin queens with the drug treatments.**

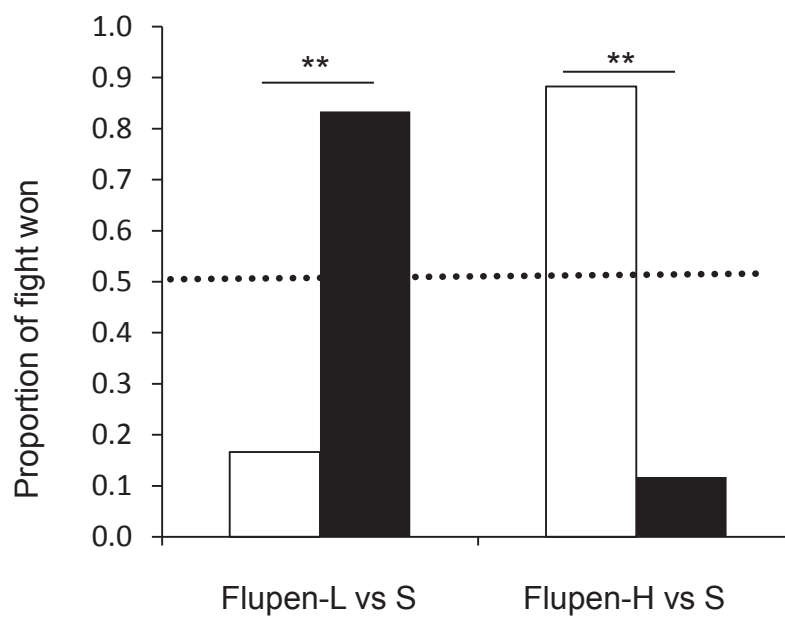
Treatments	N	Fight initiation	Fight duration
		Mean $\pm$ SD	Mean $\pm$ SD
Saline vs. saline	10 pairs	23.8 $\pm$ 17.09	338.4 $\pm$ 427.27
Low-level DA vs. saline	10 pairs	31.9 $\pm$ 41.63	235.3 $\pm$ 142.31
High-level DA vs. saline	10 pairs	40.3 $\pm$ 28.96	209.4 $\pm$ 343.33
Low-level flupenthixol vs. saline	18pairs	41.8 $\pm$ 36.73	223.1 $\pm$ 264.41
High-level flupenthixol vs. saline	17 pairs	38.4 $\pm$ 34.78	276.3 $\pm$ 271.27

SD: Standard division

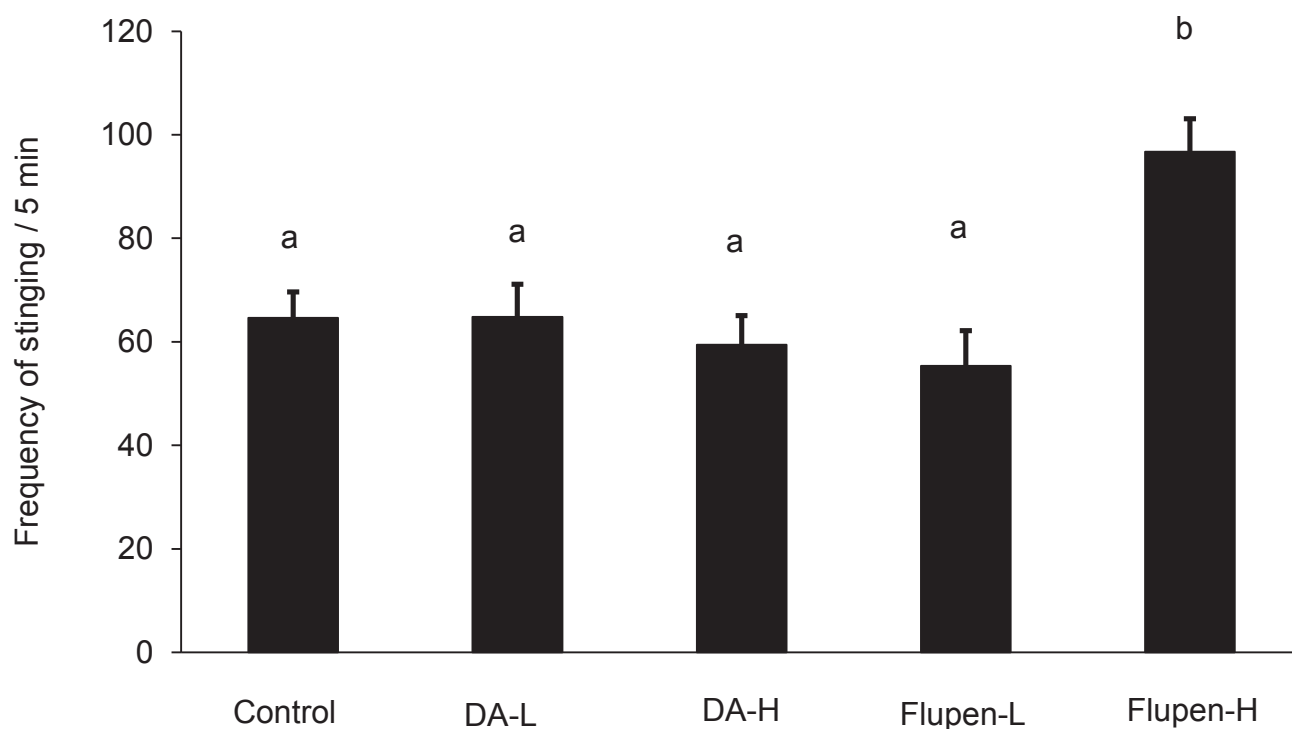




**Fig. 1-3. The effect of DA on fight. The proportion of winning in high-level DA ( $1.0 \times 10^{-2}$  M) (open bar) vs. saline (closed bar) ( $n = 10$  pairs) and low-level DA ( $1.0 \times 10^{-3}$  M) (open bar) vs. saline-injected virgin queens (closed bar) ( $n = 10$  pairs). DA-L: low-level DA ( $1.0 \times 10^{-3}$  M) injected. DA-H: high-level DA ( $1.0 \times 10^{-2}$  M) injected. S: Saline injected. NS: Not significant**



**Fig. 1-4. The effect of DA receptor antagonist on the fight. The proportion of winning in high-level DA receptor antagonist-injected (open bar) vs. saline-injected virgin queens (closed bar) (n = 18 pairs) and low-level DA receptor antagonist-injected (open bar) vs. saline-injected virgin queens (closed bar) (n = 17 pairs). Flupen-L: low-level DA receptor antagonist flupenthixol ( $1.0 \times 10^{-3}$  M) injected. Flupen-H: high-level DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected. S: Saline injected. \*\*:  $p < 0.01$**



**Fig. 1-5. The effect of DA and DA receptor antagonist on the stinging behavior of virgin queens. The frequency of stinging in treatments injected one-day-old virgin queens. The graph indicates the mean of stinging with standard error. Letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference. DA-L: low-level DA ( $1.0 \times 10^{-3}$  M) injected (n = 10). DA-H: high-level DA ( $1.0 \times 10^{-2}$  M) injected (n = 11). Flupen-L: low-level DA receptor antagonist flupenthixol ( $1.0 \times 10^{-3}$  M) injected (n = 12). Flupen-H: high-level DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected (n = 13). Control: Saline injected (n = 20).**

## 1.5 Discussion

In this part, I studied the neurophysiological role of DA for the fighting and aggression of honey bee virgin queens. DA injection did not affect the fighting and stinging behavior in queens. DA receptor antagonist flupenthixol, however, affected these behaviors; it suggests that DA plays a role in the regulation of the fighting and aggression that are associated with survival for becoming the next reproductive member of the colony in virgin queens, although a blockade of DA does not always inhibit these behaviors.

### 1.5.1 Influence on fighting behavior:

DA injection in one-day-old virgin queen did not affect the fighting behavior, although it has been reported that DA affect fighting in several insects (Alekseyenko *et al.*, 2013; Rillich and Stevenson, 2014; Szczuka *et al.*, 2013). Because one-day-old virgin queens have high brain DA levels (Harano *et al.*, 2005, 2008), their DA receptors might be saturated with endogenous DA, which may make it likely for DA injection to have no effect on fighting.

To find the effect of DA on fighting behavior, I injected DA receptor antagonist flupenthixol, which can block D1-like receptors (mainly AmDOP2 but also AmDOP1) in honey bees (Beggs *et al.*, 2011; Blenau *et al.*, 1998; Mustard *et al.*, 2003). Surprisingly, two different concentrations of flupenthixol had opposite effect on fighting behavior. The injection of flupenthixol at low concentrations ( $1.0 \times 10^{-3} \text{M}$ ) decreased the winning probability of virgin queens, whereas high concentration injections ( $1.0 \times 10^{-2} \text{M}$ ) increased it. The weak blockade of DA receptors by flupenthixol at low concentrations ( $1.0 \times 10^{-3} \text{M}$ ) can inhibit fighting behavior, which is consistent with the inhibition of locomotor activities by flupenthixol at high

concentration ( $1.0 \times 10^{-2} \text{M}$ ) that was previously reported (Harano *et al.*, 2008). However, with same concentration to that of Harano *et al.* (2008) ( $1.0 \times 10^{-2} \text{M}$ ) an opposite result was found. This suggests that the effect of flupenthixol on the outcome of fighting cannot be explained only by its modulatory effect on locomotion. I do not know why the opposite effects were exerted by injections at higher concentrations. Non-linear effects of the concentration of this substance were also reported by Mustard *et al.* (2010). They found that injections of  $5 \times 10^{-6} \text{M}$  and  $5 \times 10^{-4} \text{M}$  flupenthixol decreased the locomotor behavior during a period of 25 minutes after injection into honey bee workers; however,  $5 \times 10^{-3} \text{M}$  and  $5 \times 10^{-5} \text{M}$  did not affect such behaviors. The other study showed that flupenthixol has different antagonistic potency for AmDOP1 and AmDOP2 (Mustard *et al.*, 2003), indicating that different concentrations of flupenthixol are required to differentially block activities of these receptors. In addition, flupenthixol has been suggested to have an effect on the octopamine receptor (AmOA1) in honey bees (Beggs *et al.*, 2011). Octopamine controls fighting behavior and aggressiveness in many insects including honey bee workers (Hunt, 2007). For examples, OA promotes the escalation and maintenance of aggression once it started in crickets (Rillich and Stevenson, 2015), Abdominal injection of OA decreased the stinging response ratio, but increased occurrence of full sting extension (Burrell and Smith, 1995). Thus, the blockade of these receptors to different extents, depending on the concentration, might explain opposite effects of flupenthixol on the outcome of fighting at two concentrations. The strong blockade of DA receptors or other amine receptors promoting the fighting behavior suggests the existence of inhibitory neural systems of fighting behavior mediated by DA and other amines like octopamine. AmDOP2 that is blocked by a lower concentration of flupenthixol might have a function to drive fighting, and either AmDOP1 or AmOA1 might inhibit fighting.

### 1.5.2 Influence on stinging response:

Stinging is a crucial process in queen-queen fights because a queen who successfully stings her opponent wins the fight, as Gilley (2001) observed. On the other side, stinging can be an indicator of escalated aggression in social insects (Santoro *et al.*, 2015). Therefore, I measured the stinging behaviors of one-day-old virgin queens injected with DA and flupenthixol to investigate the difference of results to that of fighting behavior. Similar to the fighting experiment, I found that DA injection did not affect stinging behavior. Flupenthixol at high concentrations ( $1.0 \times 10^{-2} \text{M}$ ) increased the frequency of stinging response (similar to that of fighting). However, at a low concentration ( $1.0 \times 10^{-3} \text{M}$ ), the effect was not statistically significant. These results were not consistent with my prediction that DA injection promotes stinging behavior while its antagonist exerts the opposite effect. Nonetheless, a modification of this behavior caused by DA receptor antagonist suggests that DA is involved in the control of aggression in honey bee virgin queens (discussed in 1.5.1).

## 1.6 Conclusion

In summary, this chapter showed the importance of DA signaling in the regulation of fighting and aggressive behavior in honey bee virgin queens by injecting a DA receptor blocker, flupenthixol. This treatment at lower concentration decreased probability of winning the fight, whereas at high concentration increased probability of winning the fight and increased the intensity of stinging behavior. These opposite responses might result from different antagonistic potency of several types of dopamine receptors or other monoamine receptors in response to different concentrations of flupenthixol. Further studies are needed to examine the mechanism how DA signaling regulates the fighting and aggressive behaviors of honey bee queens and DA receptor involvement in regulation of the fighting and aggressive behaviors.

## **Chapter 2**

### **The influence of dopamine on the flight behavior of honey bee virgin queens**



## 2.1 Abstract

DA, one of biogenic amines, has been suggested to regulate the physiology and behavior in the virgin queens in honey bees. The involvement of DA in the high locomotor activity and high aggressiveness against rival sister-queens in the virgin queens has been reported. In the current chapter, I tested other roles of DA in the behavior of honey bee virgin queens. I investigated the effect of DA on flight behavior of 6-day-old virgin queens with a flight mill by injection of DA and a DA receptor antagonist flupenthixol. The injection of DA did not affect the flight initiation, flight distance, flight duration and the flight velocity. However, the injection of flupenthixol significantly delayed the initiation of flight and decreased the flight performance; the distance to flight, duration of flight and the flight velocity. These results suggest that DA regulates the flight behavior of the virgin queens. The role of DA on flight behavior for mating may be conserved in the virgin queens as well as drones in honey bees previously reported.

## 2.2 Introduction

Mating for females is a critical event for transition from one behavioral states seeking or choosing their own mate or dispersing to avoid inbreeding to the other states producing eggs and ovipositing. Limited energy in the female body and time of their lives is allocated intensively to locomotion including flight before mating, but to production of eggs after mating. The trade-off between locomotion and egg production has been reported in several insects, which is based on the drastic behavioral changes by mating. These changes in insects like butterflies (Obara *et al.*, 2011), moths (Saveer *et al.*, 2012), fruit flies (McGraw *et al.*, 2008), ants (Schrempf *et al.*, 2005) and bees (Harano *et al.*, 2007; Kocher *et al.*, 2010) have been reported. However, it is unclear how these changes occur and how each behavioral state kept is. Biogenic amines are the most likely candidates that can change the physiological states by mating or maintain each behavior before and after mating (Harano *et al.*, 2005, 2008; Obara *et al.*, 2011; Aonuma and Watanabe, 2012).

Drastic behavioral changes by mating have been known in honey bee queens. The virgin queens have a high locomotor activity (Harano *et al.*, 2007) and destroy queen cells with younger sister-queens (Gilley and Tarpy, 2005; Winston, 1987). If a sister-queen emerges before destruction, the lethal fight occurs between the queens. The surviving queen performs mating flights as she gets sexually matured (one week after emergence), flies several kilometers away from the colony to the mating area, mates in mid-air (Zmarlicki and Morse 1963), and then returns directly to the colony. After mating, the queen develops her ovaries and starts laying eggs. Mated queens show decreased locomotor activity (Harano *et al.*, 2007) and never fly except when they leave the nest to establish new colony (swarming) in the other year (Gilley and Tarpy, 2005; Winston, 1987). High levels of brain DA might facilitate mating flights by activating

motor systems in virgin queens as DA enhances locomotor activity in various animals (Chase *et al.*, 2004; Draper *et al.*, 2007; Lima and Miesenbock, 2005; Rhodes *et al.*, 2005), including honey bees (Beggs *et al.*, 2007; Mustard *et al.*, 2010; Akasaka *et al.*, 2010; Harano *et al.*, 2008).

In chapter 1 I examined the effect of DA on lethal fight and aggression in young virgin queens, but it does not explain why high DA levels are observed in matured virgin queens (Harano *et al.*, 2005, 2008). In this chapter, I focus on the flight behavior that may be associated with the brain DA levels in honey bee queens. The role of DA in flight behavior has been reported in drones (Mezawa *et al.*, 2013). Furthermore, similar effect is known in another bee; large carpenter bees (Sasaki and Nagao, 2013); moth (Claassen and Kammer, 1986) and locust (Buhl *et al.*, 2008). Therefore, I hypothesized that DA regulates the flight behavior in honey bee virgin queens. To examine this hypothesis, I injected DA and DA antagonist into abdomen of virgin queens, and measured the time to flight initiation and the flight performance including flight distance, flight duration and the flight velocity by using a flight mill.

## 2.3 Materials and Methods

### 2.3.1 Queen rearing:

Queens were reared by using same method as chapter 1 (1.3.1).

### 2.3.2 Injection of drugs:

To examine the effect of DA on flight behavior of virgin queens, similar to chapter 1 (1.3.2) dopamine hydrochloride (Sigma Aldrich) and flupenthixol dihydrochloride (European pharmacopeia), as a DA receptor antagonist, were diluted by honey bee saline to  $1.0 \times 10^{-3}$  M or  $1.0 \times 10^{-2}$  M. These solutions were then injected into the abdomen of 6-days-old virgin queens. The control queens were injected with the same amount of saline.

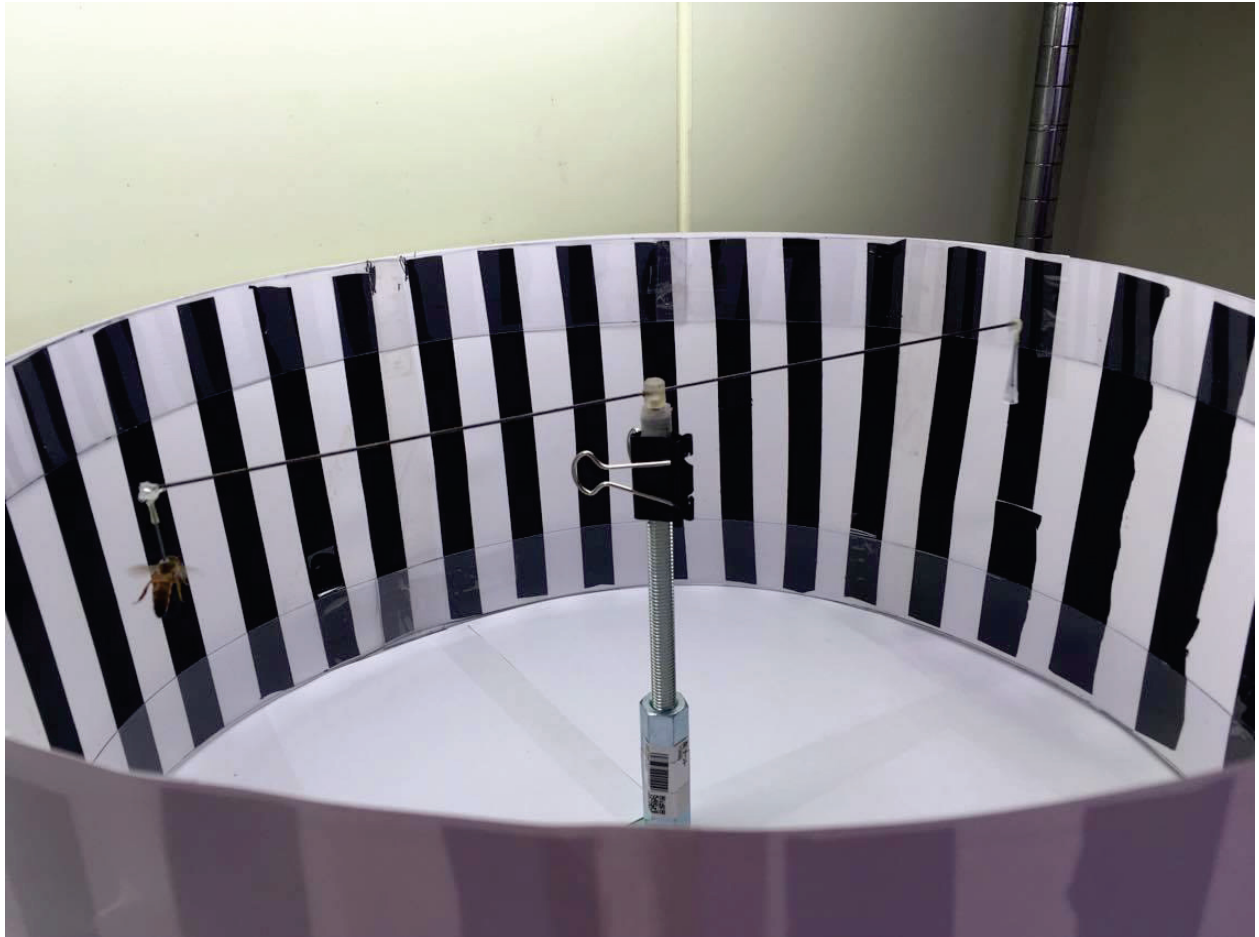
### 2.3.3 Observation of flight initiation:

After injection of drugs, virgin queens were kept in red-covered petri dishes for 20 minutes. Then, the petri dishes were opened. The duration between open of the petri dish to the commencement of flight by virgin queen, defined as the time to flight initiation, was recorded (within 5 minutes). Experiments were performed under room lights at  $431 \pm 12$  lux and  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Several virgin queens did not initiate flight during observations but, there was no significantly difference among groups (Fisher's exact test:  $\chi^2 = 0.288$ ,  $p = 0.99$ ). Therefore, only virgin queens flew within 5 minutes were used for the analysis.

### 2.3.4 Measurement of flight performance by flight mill:

For the observation of flight performance, virgin queens were kept in red-covered petri dishes for 20 minutes after injection of drugs ( $1.0 \times 10^{-2}$  M DA or Flupenthixol). Then, each virgin queen was attached with a pin like nail that was glued (Aron alpha) to the dorsal side of thorax to the 31.8 cm diameter (one circumference of flying covers 1 meter) flight mill (Fig.2-1). To make balance, weight (micropipette tip with a pin like nail) was glued on the other arm of flight mill. The flight mill was surrounded by a striped paper wall, and set under a constant temperature and lightening. I measured the flight performance of all queens (control, agonist, and antagonist) on one flight mill with video recording (Fig. 2-1).

Because the amount of energy sources that bees possess should have a strong effect on flight performance of honey bees (Gmeinbauer and Crailsheim, 1993), I let virgin queens fly twice. In the first flight, the queen was stimulated to fly to completely lose the energy. Every time the queen stops fluttering, she was stimulated to restart fluttering by giving a small paper ball to land and removing until the movements of wings were very weak and the virgin queen could no longer move the arm of the flight mill. Soon after the first flight, each virgin queen was fed on 10  $\mu$ l of 2M glucose solution by a micro pipet. After a resting period for 5 minutes, the queen was attached again to the flight mill to perform the second flight. The queens were weighted on an analytical balance to the nearest 0.1 mg before and after feeding to confirm that the glucose solution was successfully fed to the queens. The queens were also weighted after the second flights to measure the loss of weight by the second flight. Only the virgin queens that accepted all 10  $\mu$ l glucose were used in the experiment. All flights were recorded by video camera, and then the flight distance, duration of flight and average of flight velocity (distance/duration) were calculated.



**Fig. 2-1. Flight mill. A flight mill attached virgin queen for observing flight.**

### 2.3.5 Statistical analysis:

Generalized linear mixed model (GLMM) was used to examine whether DA and a DA antagonist flupenthixol affected the flight behavior in both flight initiation and flight performance.

Before performing the GLMM, I checked the distribution of variables with Shapiro test. Then while performing GLMM, I applied a Gamma distribution for the data that were not normally distributed and a Gaussian distribution for the normally distributed data.

In the observation of flight initiation, the duration until flight initiation was defined as response variable, the treatments and body weight as explanatory factors, and the larvae source colonies and the queen-less grafted colonies for queen rearing as random factors. To evaluate the effects of treatment, each treatment group was compared with the control group by GLMM and then the *p*-values were corrected by using Bonferroni *p*-value adjustment.

In the observation of flight performance, the flight distance, flight duration and the average of flight velocity were defined as response variables, the treatments and body weight as explanatory variables, and larvae source colonies and the queen-less grafted colonies for queen rearing as random factors. To find the effect of drugs, I compared each treatment group with the control group.

To examine statistical differences among experimental groups, I applied likelihood ratio tests (LRT) at a 5 % significant level where *p*-values were corrected by Bonferroni *p*-value adjustment. The GLMM test was performed using lmer function with the Lme4 package in R version i386.3.4.2 for windows.

## 2.4 Results

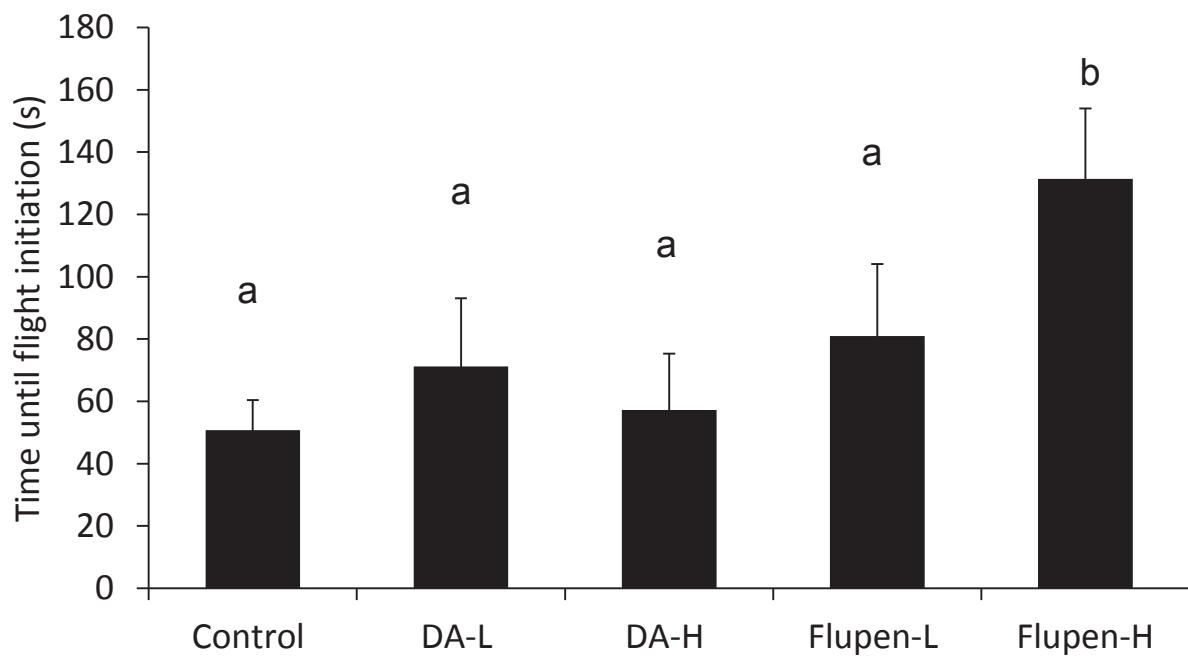
### 2.4.1 Flight initiation:

Drug injection significantly affected the flight initiation in virgin queens (Table 2-1). The initiation of flight was significantly delayed in high-level flupenthixol ( $1.0 \times 10^{-2} \text{M}$ ) injected group in comparison to control (GLMM: LRT with Bonferroni  $p$ -value adjustment: d.f = 1,  $\chi^2 = 9.31$ ,  $p < 0.01$ ). No significant difference was found in other treatment groups to control (low-level DA, d.f = 1,  $\chi^2 = 1.88$ ,  $p = 0.676$ ; high-level DA, d.f = 1,  $\chi^2 = 0.105$ ,  $p = 1$ ; low-level flupenthixol, d.f = 1,  $\chi^2 = 1.98$ ,  $p = 0.64$ , Fig. 2-2). The body weight did not affect the flight initiation (Table 2-1).



**Table 2-1. The effect of treatments and body weight on the flight initiation.**

<b>Variables</b>	<b>d.f</b>	<b><math>\chi^2</math></b>	<b><math>p</math>-value</b>
Treatment	4	9.833	0.04333
Body weight	1	0.4975	0.4806



**Fig. 2-2. The effect of DA and DA receptor antagonist on flight initiation of virgin queens.**

The graph indicates the mean of the time until flight initiation with standard error. Letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference. DA-L: low-level DA ( $1.0 \times 10^{-3}$  M) injected (n = 14). DA-H: high-level DA ( $1.0 \times 10^{-2}$  M) injected (n = 15). Flupen-L: low-level DA receptor antagonist flupenthixol ( $1.0 \times 10^{-3}$  M) injected (n = 14). Flupen-H: high-level DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected (n = 16). Control: Saline injected (n = 19).

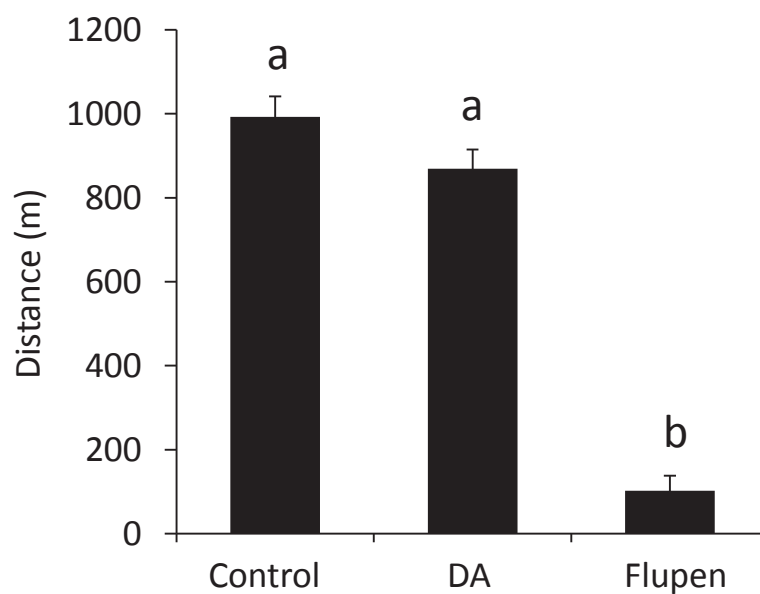
### 2.4.2 Flight performance:

The flight performance was significantly different among treatments. All three parameters that include flight distance, flight duration and the flight velocity were significantly different among groups (Table 2-2). Multiple comparisons showed that the flight distance (GLMM: LRT with Bonferroni  $p$ -value adjustment: d.f = 1,  $x^2 = 39.74$ ,  $p < 0.001$ , Fig. 2-3), flight duration (d.f = 1,  $x^2 = 39.83$ ,  $p < 0.001$ , Fig. 2-4) and flight velocity (d.f = 1,  $x^2 = 27.71$ ,  $p < 0.001$ , Fig. 2-5) were significantly lower in flupenthixol injected virgin queens than in control. However, all three parameters in DA injected individuals were not significantly different from the control (flight distance, d.f = 1,  $x^2 = 4.43$ ,  $p = 0.07$ ; flight duration, d.f = 1,  $x^2 = 0.262$ ,  $p = 1$ ; flight velocity, d.f = 1,  $x^2 = 0.53$ ,  $p = 0.94$ ; Fig. 2-3, 2-4, 2-5). There was no effect of body weight on flight distance, flight duration, but there was a negative correlation between body weight and the average of flight velocity (Table 2-2).

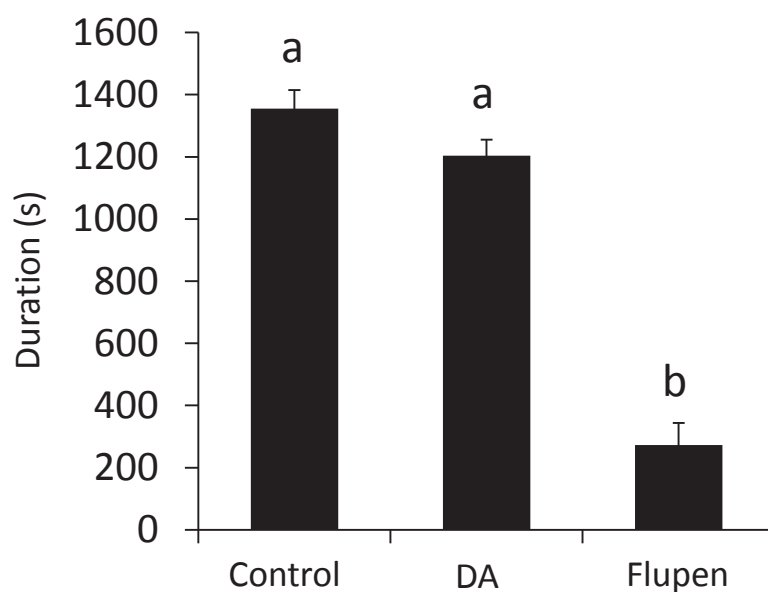
The weight loss in second flight was measured. The weight loss was significantly different among groups (GLMM: LRT: d.f = 2,  $x^2 = 19.19$ ,  $p < 0.001$ ). The flupenthixol injected group had significantly lower weight loss than control (GLMM: LRT with Bonferroni  $p$ -value adjustment: d.f = 1,  $x^2 = 13.80$ ,  $p < 0.001$ , Fig. 2-6). However, there was no significant difference between DA injected and control (GLMM: LRT with Bonferroni  $p$ -value adjustment: d.f = 1,  $x^2 = 0.09$ ,  $p = 0.76$ , Fig. 2-6).

**Table 2-2. The effect of treatments and body weight on the flight performance.**

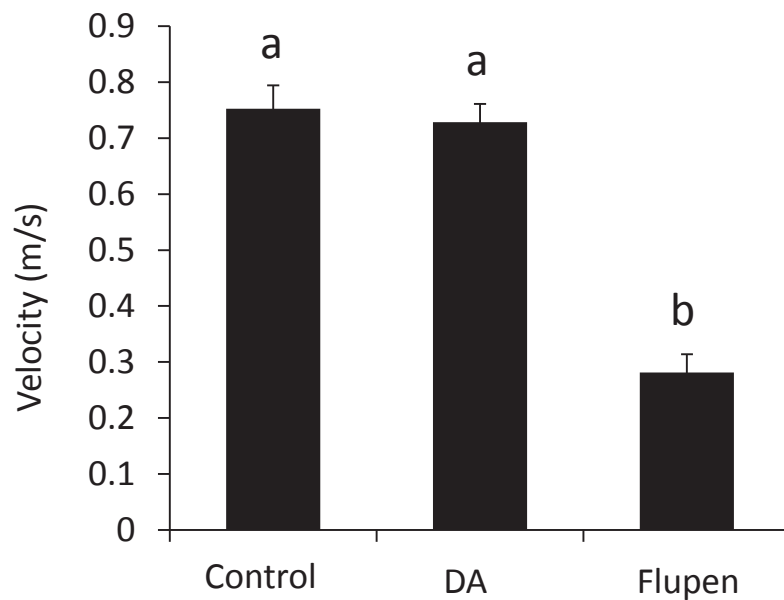
Response variables	Explanatory variables	d.f	$x^2$	$p$ -value
Distance	Treatment	2	56.76	<0.001
	Body weight	1	0.0092	0.92
Duration	Treatment	2	57.25	<0.001
	Body weight	1	0.27	0.60
Velocity	Treatment	2	42.07	<0.001
	Body weight	1	0.923	0.34



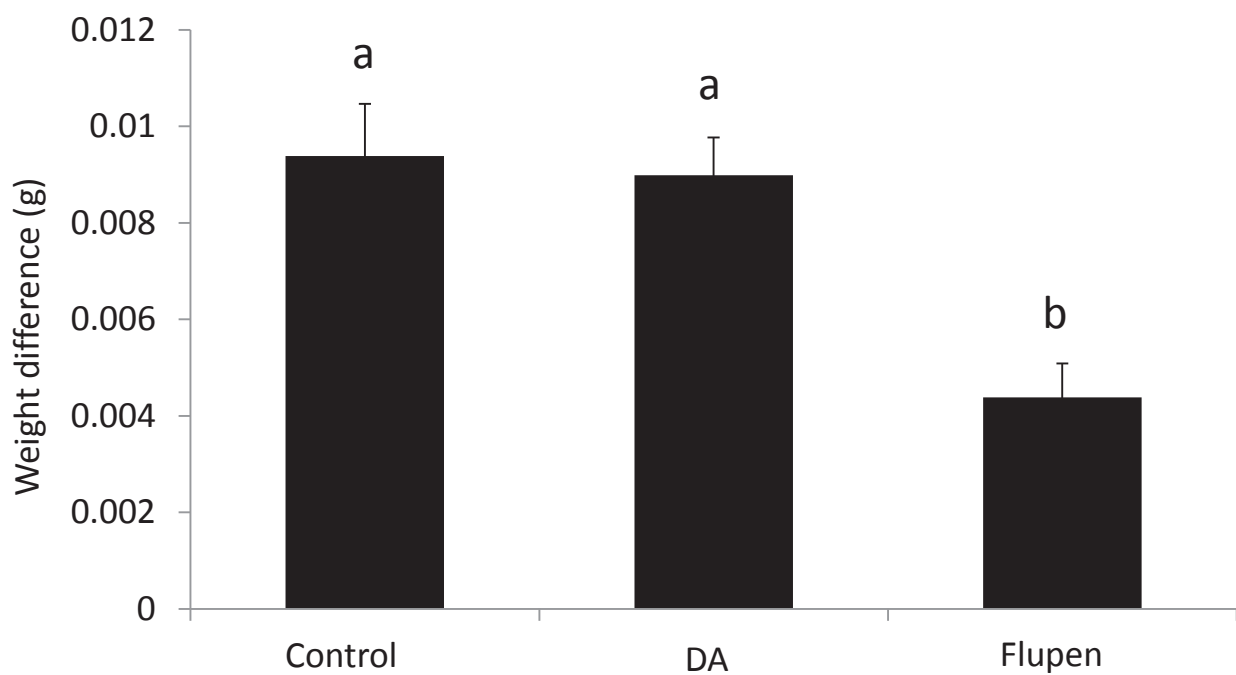
**Fig. 2-3. The effect of DA and DA receptor antagonist on the flight distance. The graph indicates the mean with standard error. Letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference. DA: dopamine ( $1.0 \times 10^{-2}$  M) injected (n = 21). Flupen: DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected (n = 22). Control: Saline injected (n = 20).**



**Fig. 2-4. The effect of DA and DA receptor antagonist on the flight duration. The graph indicates the mean with standard error. Letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference. DA: dopamine ( $1.0 \times 10^{-2}$  M) injected (n = 21). Flupen: DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected (n = 22). Control: Saline injected (n = 20).**



**Fig. 2-5.** The effect of DA and DA receptor antagonist on the average of flight velocity. The graph indicates the mean with standard error. Letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference. DA: dopamine ( $1.0 \times 10^{-2}$  M) injected (n = 21). Flupen: DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected (n = 22). Control: Saline injected (n = 20).



**Fig. 2-6.** The body weight difference of virgin queens between before (after feeding) and after second flight. The graph indicates the mean with standard error. Letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference. DA: dopamine ( $1.0 \times 10^{-2}$  M) injected (n = 21). Flupen: DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) injected (n = 22). Control: Saline injected (n = 20).



## 2.5 Discussion

In the current chapter, I studied the neurophysiological role DA on flight behavior in honey bee virgin queens. I showed that DA injection did not affect the flight initiation, flight distance, flight duration and the flight velocity. However, injections of a DA receptor antagonist flupenthixol delayed the initiation of flight, and decreased the flight distance, flight duration and the average of flight velocity. These results suggest that DA is involved in the regulation of flight behavior in virgin queens in the honey bee.

Role of DA on flight behavior seems to be conserved between sexes in the honey bee. The results of this chapter indicate that DA is involved in the regulation of flight behavior in virgin queens. Similarly, the involvement of DA in the regulation of flight is also reported in drones (Mezawa *et al.*, 2013). In drones, DA regulates flight behavior under the control of juvenile hormone (JH; Mezawa *et al.*, 2013). In queens, Wegener *et al.* (2013) reported that JH titers decrease following mating, same as decrease of the brain DA levels (Harano *et al.*, 2005, 2008). Given the parallel changes between JH and DA, the DA system regulated by JH might be shared between queens and drones. However, another study shows that JH increases following mating in queens (Fluri *et al.*, 1981) while DA levels decreases (Harano *et al.*, 2005, 2008), suggesting that DA is not under control of JH in queens. Study about the relationship between JH and DA in queens remains to be examined.

DA may play a basic role in the activation of locomotor system. DA affects the locomotion in various kinds of animals (Chase *et al.*, 2004; Draper *et al.*, 2007; Lima and Miesenbock, 2005; Rhodes *et al.*, 2005) including honey bees queens (Harano *et al.*, 2008), drones (Akasaka *et al.*, 2010) and workers (Beggs *et al.*, 2007; Mustard *et al.*, 2010), suggesting

that DA affects the locomotor system in both castes and sexes of the honey bee. Three subtypes of DA receptors have been identified in honey bees (AmDOP1, AmDOP2 and AmDOP3). Among these receptors, AmDOP2 has been shown to modulate the locomotion in workers (Mustard *et al.*, 2010). Flupenthixol, a DA receptor antagonist used in this study, blocks AmDOP2 stronger than AmDOP1 and AmDOP3 (Blenau *et al.*, 1998; Mustard *et al.*, 2003; Beggs *et al.*, 2011). Because flight needs activated motor system (Lehmann and Bartussek, 2017; Dickinson *et al.*, 1998), it is likely that the role of DA on flight activity in virgin queens found in this study (similar to the modulation of motor behavior in workers) is mediated by AmDOP2.

Flupenthixol-injected queens lost only 4 mg of body weight while control and DA-injected queens lost more than twice. It appeared that the flupenthixol-injected queens stopped flying before ran out sugar solution as fuel. These results suggest that queens cannot fly for a long time when DA signaling is depressed even if they have sufficient amount of fuel.

The abdominal injection of DA in virgin queens did not affect the flight initiation and flight performance. Whilst previous studies found an effect of DA abdominal injection on the flight behavior in drones (Mezawa *et al.*, 2013; Sasaki and Nagao, 2013), results shown in chapter 1 revealed that DA abdominal injection did not affect the fighting and stinging behaviors in virgin queens (chapter 1). I speculated that it is due to the presence of large amount of endogenous DA in the brain of virgin queens (Harano *et al.*, 2005, 2008), so that the most of receptors are bounded with the endogenous DA. Thus, the injected DA could not bind to the receptors, and did not affect the flight behavior.

## 2.6 Conclusion

In conclusion, I found the involvement of DA in flight behavior in honeybee queens. This finding partially explains why virgin queens show higher DA levels than mated queens (Harano *et al.*, 2005, 2008). Activating DA receptors is likely to facilitate long flights and increase a chance of mating. Further studies are needed to investigate how DA regulates the flight behavior and the effect of DA on queen's mating success.

## **Chapter 3**

### **The influence of dopamine and octopamine on the flight initiation in workers**

### 3.1 Abstract

Biogenic amines like DA and OA act as neuroactive substances, affecting physiology and behavior in both vertebrates and invertebrates. Several studies have investigated the effects of DA and OA on various behaviors and the division of labor in workers in honey bees. In the present chapter, I investigated the effects of DA and OA on the promotion of flight initiation in foraging workers in the European honey bee *Apis mellifera*. I injected different doses ( $10^{-3}$  M and  $10^{-2}$  M) of DA, OA, the DA receptor antagonist flupenthixol, and the OA receptor antagonist epinastine into the abdomen of foragers, and then measured the time to flight initiation. Injections of both flupenthixol and epinastine into foragers caused a significant delay in the onset of flying in comparison to control foragers. However, the foragers injected with DA or OA did not show any significant delay compared to the controls. The results suggest that strong inhibition of DA or OA signaling may affect the motor system for flight behavior which is necessary for foraging in workers.

## 3.2 Introduction

Flight is relevant to the age-related division of labor in honey bee workers. In general, worker bees can be divided into four temporal castes on the basis of age: the cell cleaners (1-4 days old), the nurses (4-12 days old), the middle-aged bees (12-21 days old) that perform a range of tasks such as nest building, receiving and processing food, and guarding. These young bees do not often fly, comparing to older ones. The older bees (21+ days old) frequently perform flights to forage outside of the colony (Winston, 1987). The foraging success depends on the flying ability of foragers; efficient resource acquisition can have profound effects at the colony level and affects the pollination service provision (Higginson *et al.*, 2011; Becher *et al.*, 2014). The transition from one temporal caste to another based on age is plastically controlled by social environment (Johnson, 2010) and internal factors (Robinson and Vargo, 1997).

Schulz and Robinson (1999) and Wagener-Hulme *et al.* (1999) have shown a correlation between brain level biogenic amines, particularly OA, and the age-related division of labor. Later, Schulz and Robinson (2001) and Schulz *et al.* (2002) found that OA regulates the onset of foraging behavior. OA is also related to locomotor behavior (Fussnecker *et al.*, 2006), fanning behavior (Cook *et al.*, 2017) and dance behavior (Barron *et al.*, 2007). Thus, it appears as though OA is involved in flying or flight motivation in workers. Another biogenic amine, DA modulates behaviors such as locomotor activity (Mustard *et al.*, 2010) and recruitment behavior in dances (Božič and Woodring, 1998), whereas modulations of flight behavior by DA in workers remain to be unclear.

Honey bee queens typically perform flights when they are virgin and the brain level DA is higher than the time they do not fly (after mating) (Harano *et al.*, 2005, 2008; Winston, 1987).

Same as queens the typical flight performers of honey bee workers also have higher amount of brain level DA than the hive task performers (Harris and Woodring, 1992). The findings of chapter 2 indicate that the blockade of DA suppress the flight motivation and flight performance. Therefore, in here I assumed that DA may affect the flying behavior in workers, too. In honey bee workers it has been reported that similar to DA the brain level of OA is also higher in foragers than the hive task performers (Harris and Woodring, 1992; Wagener-Hulme *et al.*, 1999). Similar effects of DA and OA on flight initiation in drones have been reported (Mezawa *et al.*, 2013). Here, I tested whether this similarity conserved in workers too or not.

In this chapter, to examine the regulatory effect of DA and OA on flight motivation in honey bee workers, I measured the duration of flight initiation of pollen foraging workers which were injected DA, OA and their antagonists.

## 3.3 Materials and methods

### 3.3.1 Bees:

Pollen foragers returning from the field were collected from 5 colonies for the DA experiments and 4 colonies for the OA experiments. Each colony contained more than 10 comb frames. The DA experiments were performed in May 2016, and the OA experiments were performed in May 2017. In both experiments, foragers were collected between 10:30 am and 12:30 pm and fed a 50% sugar solution until drug injections. I performed experiments between 1:00 pm and 5:00 pm on the same day that the bees were collected from the colonies.

### 3.3.2 Injection of biogenic amines and receptor drugs:

To determine the effects of DA and OA on flight initiation in worker bees, dopamine hydrochloride (Sigma Aldrich), flupenthixol (European pharmacopeia) (a DA receptor antagonist), octopamine hydrochloride (Sigma Aldrich), and epinastine hydrochloride (Tokyo chemical industry) (an OA receptor antagonist) were diluted with honey bee saline to  $1.0 \times 10^{-3}$  M (low concentration) or  $1.0 \times 10^{-2}$  M (high concentration) and injected (1  $\mu$ L/bee) into the abdomen of forager bees using a Hamilton syringe (Hamilton Company) (Mezawa *et al.*, 2013; Sasaki and Nagao, 2013). The control bees were injected with an equal amount of saline. All bees were anesthetized on ice for 5 minutes prior to injection. Three hundred and seven bees were used in the experiments.



### **3.3.3 Observation of flight initiation:**

Behavioral experiments were performed in the laboratory under room lights at  $449 \pm 11$  lux and  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . The observation procedure was similar to that of chapter 2 (2.3.3).

### **3.3.4 Data analysis:**

I performed Kruskal-Wallis tests followed by Dunn's multiple comparison to compare the time to flight initiation between the treatments, using R version 3.6.3 for windows.

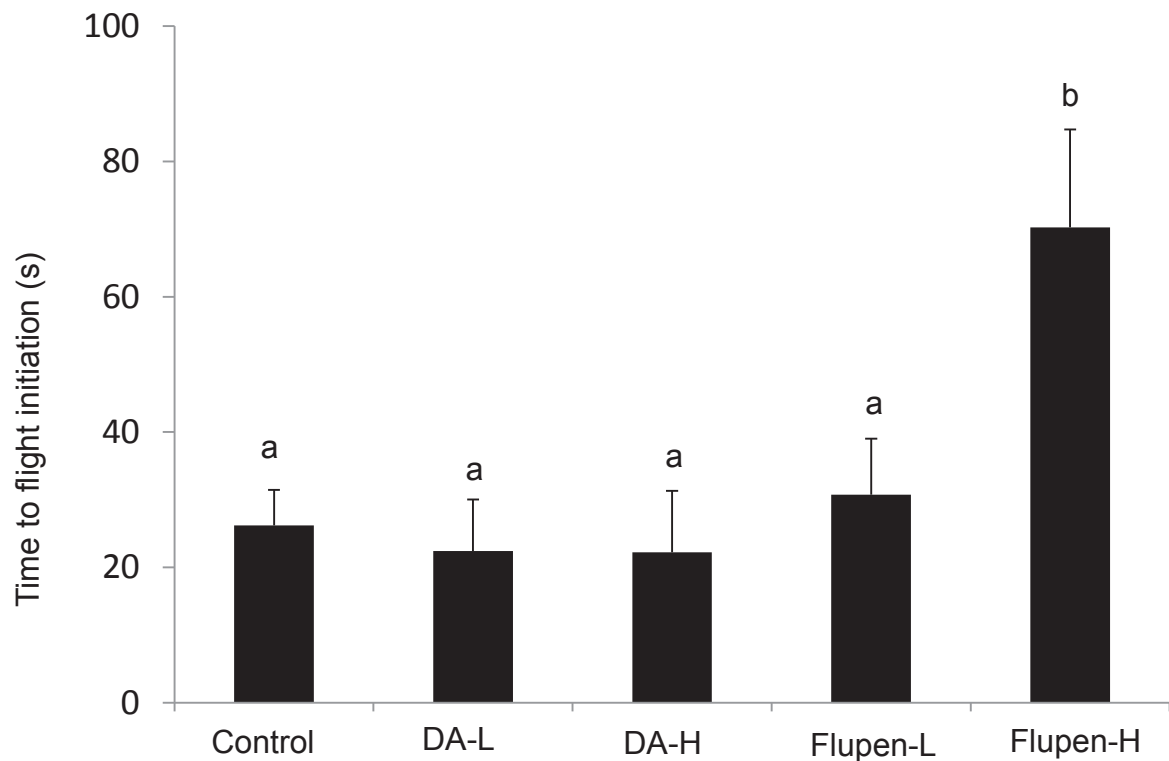
## 3.4 Results

### 3.4.1 Dopamine and its receptor antagonist:

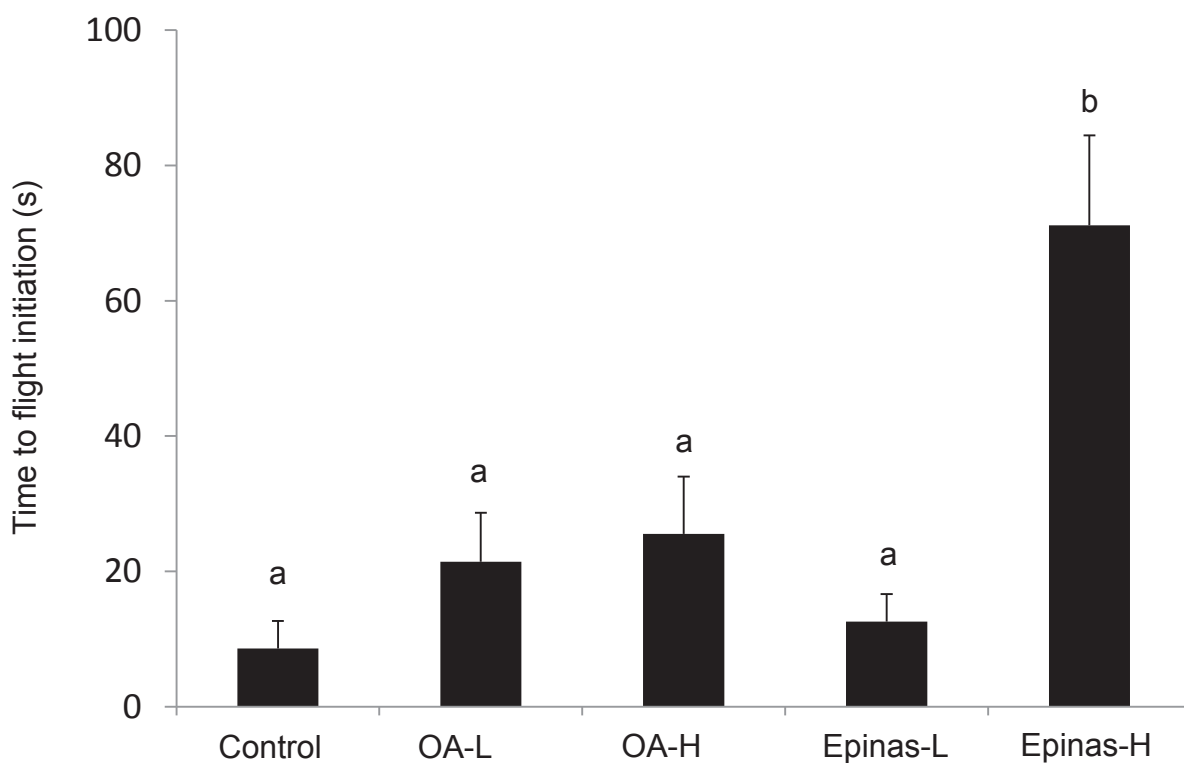
I found a significant difference in the time to flight initiation between treatments (Kruskal-Wallis test:  $df = 4$ ,  $\chi^2 = 29.55$ ,  $p < 0.001$ ). The time to flight initiation was much longer in the bees injected with high concentration flupenthixol than in the other groups (Fig. 3-1). Dunn's post hoc analysis revealed that the time to flight initiation was significantly longer in the high concentration flupenthixol-injected bees than in the control bees ( $p < 0.01$ ). There were no significant differences in the other groups comparing to control group: low concentration DA ( $p = 0.644$ ), high concentration DA ( $p = 0.055$ ), low concentration flupenthixol ( $p = 0.326$ ).

### 3.4.2 Octopamine and its receptor antagonist:

There was a significant difference in the time to flight initiation between treated groups (Kruskal-Wallis test:  $df = 4$ ,  $\chi^2 = 32.73$ ,  $p < 0.001$ ). The time to flight initiation was significantly higher in the high concentration epinastine-injected group than in control (Fig 3.2). Dunn's post hoc analysis showed that the time to flight initiation was significantly different between the control vs. the high concentration epinastine group ( $p < 0.001$ ). There were no significant differences in the other groups comparing to control group: low concentration OA ( $p = 0.253$ ), high concentration OA ( $p = 0.585$ ), low concentration epinastine ( $p = 0.068$ ).



**Fig. 3-1. The effect of dopamine (DA) and the DA receptor antagonist flupenthixol on flight initiation.** The graph indicates the mean and standard error of the time to flight initiation. The letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference (Dunn's post hoc analysis). DA-L: low-concentration DA ( $1.0 \times 10^{-3}$  M) (n = 32). DA-H: high-concentration DA ( $1.0 \times 10^{-2}$  M) (n = 35). Flupen-L: low-concentration DA receptor antagonist flupenthixol ( $1.0 \times 10^{-3}$  M) (n = 26). Flupen-H: high-concentration DA receptor antagonist flupenthixol ( $1.0 \times 10^{-2}$  M) (n = 31). Control: Saline (n = 66).



**Fig. 3-2. The effect of octopamine (OA) and the OA receptor antagonist epinastine on flight initiation.** The graph indicates the mean and standard error of the time to flight initiation. The letters above the bars indicate statistical differences: the same letter means no statistical difference and the different letter means significant difference (Dunn's post hoc analysis). OA-L: low-concentration OA ( $1.0 \times 10^{-3}$  M) (n = 22). OA-H: high-concentration OA ( $1.0 \times 10^{-2}$  M) (n = 20). Epinas-L: low-concentration OA receptor antagonist epinastine ( $1.0 \times 10^{-3}$  M) (n = 20). Epinas-H: high-concentration OA receptor antagonist epinastine ( $1.0 \times 10^{-2}$  M) (n = 15). Control: Saline (n = 40).

### 3.5 Discussion

In this chapter, I investigated the physiological control of flight motivation in honey bee workers. Injection of epinastine (an OA receptor antagonist) delayed flight initiation, which is consistent with the findings of Fussnecker *et al.* (2006) with OA receptor antagonist mianserin. Injection of flupenthixol (a DA receptor antagonist) also delayed flight initiation. However, injection of DA and OA did not affect flight initiation. Because of the high amount of endogenous DA and OA in foragers (Harris and Woodring, 1992) which may saturate receptors, administration of additional amines might not affect the behavior. The delay in flight initiation by DA or OA receptor antagonists indicates a regulatory role of DA or OA in the motivation of flight initiation in worker bees.

Both OA and DA might regulate the age-related division of labor in worker bees (Wagener-Hulme *et al.*, 1999). OA application promotes the development of workers into foragers (Schulz and Robinson, 2001). OA is also related to the fanning behavior for thermoregulation in the colony (Cook *et al.*, 2017) and dance behavior (Barron *et al.*, 2007). These findings suggest that OA is involved in the age-related division of labor in worker bees. Our findings of inhibition on flight initiation by an OA receptor antagonist support this hypothesis, because the foraging in worker bees depends on flight (Winston, 1987). In addition to OA, DA may also be involved in the age-related division of labor. DA has been reported to control locomotor activity and ovary development in worker bees (Mustard *et al.*, 2010; Sasaki and Nagao, 2001), but no study demonstrated its involvement in age-related division of labor. The inhibition of flight initiation by a DA receptor antagonist in this study suggests a possible role of DA in the regulation of flight initiation for foraging.

Suppress of flight initiation with the blockade of DA in workers in this chapter is similar to that of chapter 2 in virgin queens. It suggests that controlling the motor system for flight by DA is similar between queens and workers. In workers, in addition to motoric activity DA involves in the regulation of reproduction, when there is no queen in the colony the brain level DA increase and acts as a stimulator of ovary development (Harris and Woodring, 1992; Sasaki and Nagao, 2001; Dombroski *et al.*, 2003). In honey bee queen also ovaries develop after mating (Tanaka and Hartfelder, 2004), but grossly the brain DA levels decrease (Harano *et al.*, 2005, 2008). It suggests that DA is not involved in the regulation of reproduction in queens. Therefore, only motoric roles of DA might be shared, but not reproductive roles.

### 3.6 Conclusion

In conclusion, the results of this study demonstrate the involvement of DA and OA in the regulation of flight motivation. The regulatory roles of both DA and OA in the promotion of flight in worker bees may indicate that not only OA, but possibly also DA, regulates the age-related division of labor. Further studies using specific receptor antagonists are needed to elucidate the effects of DA and OA on flight promotion in greater detail.

## General discussion

The biogenic amine DA as a neurophysiological factor modulates different behaviors in animals. In honey bees, the brain DA levels associate with the behavioral changes and it is shown that DA regulates honey bee behaviors. In the current studies, in chapter 1, I investigated the regulatory role of DA in controlling the fight and aggression of virgin queens. Low amount DA receptor antagonist injection caused losing the fight, but the high amount increased the aggression level and caused the fight winning. In chapter 2, I revealed the role of DA in the regulation of the flight behavior of virgin queens. DA receptor antagonist injection delayed the initiation of flight and decreased the flight distance, flight duration and flight velocity. In chapter 3, I found the effect of DA on the flight behavior of foraging workers. DA receptor antagonist injection delayed the flight initiation. Overall, these studies show that the behavioral changes or behavioral plasticity are dependent on the changes of DA levels in the brain.

In this dissertation, I followed the injection method of Mezawa *et al.* (2013) and Sasaki and Nagao (2013) that have been shown that abdominal injection of DA changes the behavior. In addition, Sasaki and Nagao (2013) reported that brain DA level increases with abdominal injection of juvenile hormone analog, methoprene. In addition, it has been reported that tyramine abdominal injection could increase the brain tyramine and octopamine level in honey bee workers (Scheiner *et al.*, 2017). These facts suggest that abdominal injection could affect DA receptors in central nervous system. Therefore, abdominal injection of flupenthixol may affect fighting and aggression and flight behavior by acting on DA receptors in central nervous system. On the other side, we cannot reject the possible acting in peripheral nerve system, because, Linn *et al.* (1994) have been found that DA injection into hemolymph increases the DA level in central nervous system and thoracic ganglia. In addition, same as decrease of DA levels in the



brain, a decrease of DA levels in the hemolymph of queens after mating also have been reported (Harano *et al.*, 2008) and Akasaka *et al.*, (2010) suggest the effect of DA in the regulation of flight behavior via acting in both central nerve system and periphery in drone. So, it maybe affects by acting on DA receptors in periphery too. Overall, the distribution of DA receptors in the periphery is not well studied than brain in honey bees.

The fight in virgin queens is lethal (only one of opponents should survive). So, virgin queens could be used as model to study about lethal fight. This is the first study that reports the regulatory role of a neurophysiological factor (DA) on the lethal fight. I found the low amount blockade of DA decrease the fight wining probability, but the high amount increased. Stinging is a determinative task for the fight wining , similar to that of fighting high amount blockade of DA increased the frequency of stinging, but low amount did not affect. In lethal fight between virgin queens in addition to using the sting they also hold each other with legs and mandibles that might facilitate the stinging. So, the low amount blockade of DA might suppress the legs and mandibles activities that caused the fight losing in treated virgin queens as previous studies reported that blockade of DA suppresses the locomotor behavior (Harano *et al.*, 2008). To prevent form a lethal fight, emerged virgin queen destroys other sister-queen cells before emergence. Virgin queen destroys sister-queen cells by chewing the queen cells with mandibles and then insert her abdomen into the cells to sting the pupa, which are present inside the cells. Examination of the sister-queen cells destruction behavior in DA and DA receptor antagonist injected virgin queens might clarify that why the opposite effects of low and high level DA receptor antagonist on lethal fight are found, because the sister-queen cell destruction is almost a motoric action even if the virgin queen is aggressive.

The findings obtained from this study demonstrates that DA regulates fight and aggression, and flight behavior in virgin queens, but the mechanism of regulation might be different or different DA receptors might be involved in the regulation of fight and aggression, and flight in virgin queens. Same amount DA antagonist injection increased the aggression level and probability of fight winning (chapter 1), but decreased the flight ability (chapter 2). As it is mentioned in general introduction, there are three subtypes of DA receptors in honey bees, AmDOP 1, AmDOP 2 and AmDOP 3, and the DA antagonist flupenthixol has been suggested to block all of these receptors (Beggs *et al.*, 2011; Blenau *et al.*, 1998; Mustard *et al.*, 2003). However, these receptors have antagonistic roles. For example: the activation of AmDOP 3 causes decrease of intracellular cAMP, on the other hand, the activation of AmDOP 1 and AmDOP 2 causes increase of intracellular cAMP (Blenau *et al.*, 1998; Humphries *et al.*, 2003; Mustard *et al.*, 2003, 2005). So, the DA receptor involvement might be specific in the regulation of these behaviors. The future studies should find which receptor is involved in the regulation of each of these behaviors. In fighting and aggression experiments I used 1-day-old virgin queens, because in natural condition usually virgin queens fighting happens in younger ages. In flight experiments I used 6-day-old virgin queens. Usually, virgin queens become mature and take mating flights around this age. Due to the findings of Harano *et al.* (2008), I cannot say the opposite effect of DA antagonist might be due to the difference in brain and hemolymph amount of DA in 1-day old virgin queens and 6-day-old virgin queens. This might be due to the difference in density and proportionality of DA receptors. However, the difference in density and proportionality of DA receptors in 1-day-old virgin queens and 6-day- virgin queens are not known yet.

The present study indicates that DA is important for motor activities in honey bees. In here I found DA regulates the flight behavior of queen (chapter 2) and workers (chapter 3), which are similar to that of drones (Mezawa *et al.*, 2013). In addition to flight, the role of DA in controlling the locomotor activity is same in queens (Harano *et al.*, 2008), workers (Beggs *et al.*, 2007; Mustard *et al.*, 2010), and drones (Akasaka *et al.*, 2010). Flight is the intensive form of motor activity and needs the motor system activation. Therefore, the similarity of the regulation of flight and locomotor activity among queens, workers and drones indicate that the DA is necessary for the activation of the motor system in honey bees. The purpose of flight is different among castes and sexes. For example: virgin queens take flights for mating, which is vital for the mating success to store enough amounts of sperm for egg fertilizing during their lives without mating again forever (Winston 1987). Therefore, the quantity, quality and genetic diversity of sperm is important for the queen fitness that has a direct effect on the colony growth and survival. The findings of this study show that DA acts as an inducer of the flight ability. So, I suggest the study of DA effects on virgin queens mating flights in colony condition and observation of mating success, quantity and quality of sperm with the degree of polyandry, and the colony growth and survival.

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## References

- Akasaka, S., Sasaki, K., Harano, K. and Nagao, T. 2010. Dopamine enhances locomotor activity for mating in male honeybees (*Apis mellifera* L.). J. Insect Physiol. 56:1160–1166.
- Alekseyenko, O.V., Chan, Y.B., Li, R. and Kravitz, E.A. 2013. Single dopaminergic neurons that modulate aggression in *Drosophila*. Proc. Natl. Acad. Sci. USA 110:6151–6156.
- Aonuma, H. and Watanabe, T. 2012. Changes in the content of brain biogenic amine associated with early colony establishment in the Queen of the ant, *Formica japonica*. PLoS ONE 7:e43377.
- Archer, J. 1988. The al biology of aggression, 1st edn. the University of Cambridge, New York.
- Barron, A.B., Maleszka, R., Vander Meer, R.K. and Robinson, G.E. 2007. Octopamine modulates honey bee dance behavior. Proc. Natl. Acad. Sci. USA 104: 1703-1707.
- Becher, M.A., Grimm, V., Thorbek, P., Horn, J., Kennedy, P.J. and Osborne, J.L. 2014. BEEHAVE: a systems model of honeybee colony dynamics and foraging to explore multifactorial causes of colony failure. J. Appl. Ecol. 51: 470-482.
- Beggs, K.T., Glendining, K.A., Marechal, N.M., Vergoz, V., Nakamura, I., Slessor, K.N. and Mercer, A.R., 2007. Queen pheromone modulates brain dopamine function in worker honey bees. Proc Natl. Acad. Sci. USA 104:2460–2464.
- Beggs, K.T., Tyndall, J.D. and Mercer, A.R. 2011. Honey bee dopamine and octopamine receptors linked to intracellular calcium signaling have a close phylogenetic and pharmacological relationship. PLoS ONE 11:e26809.

- Bernasconi, G., Ratnieks, F.L. and Rand, E. 2000. Effect of “spraying” by fighting honey bee queens (*Apis mellifera* L.) on the temporal structure of fights. *Insect. Soc.* 47:21–26.
- Blenau, W. and Baumann, A. 2001. Molecular and pharmacological properties of insect biogenic amine receptors: Lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* 48: 13–38.
- Blenau, W. and Baumann, A. 2003. Aminergic signal transduction in invertebrates: focus on tyramine and octopamine receptors. *Recent. Res. Devel. Neurochem.* 6: 225-240.
- Blenau, W., Erber, J. and Baumann, A. 1998. Characterization of a dopamine D1 receptor from *Apis mellifera*: Cloning, functional expression, pharmacology, and mRNA localization in the brain. *J. Neurochem.* 70: 15-23.
- Božič, J. and Woodring, J. 1998. Variations of brain biogenic amines in mature honeybees and induction of recruitment behavior. *Comp. Biochem. Physiol. A* 120: 737-744.
- Brandes, C., Sugawa, M. and Menzel, R. 1990. High-performance liquid chromatography (HPLC) measurement of catecholamines in single honeybee brains reveals caste-specific differences between worker bees and queens in *Apis mellifera*. *Comp. Biochem. Physiol. C* 97: 53–57.
- Buhl, E., Schildberger, K. and Stevenson, P.A. 2008. A muscarinic cholinergic mechanism underlies activation of the central pattern generator for locust flight. *J. Exp. Biol.* 211: 2346-2357.
- Burrell, B.D. and Smith, B.H. 1995. Modulation of the honey bee (*Apis mellifera*) sting response by octopamine. *J. Insect Physiol.* 41:671–680.



- Butz, V.M. and Dietz, A. 1994. The mechanism of queen elimination in two-queen honey bee (*Apis mellifera* L.) colonies. J. Apic. Res. 33:87–94.
- Carlson, N.R. 2013. Physiology of behavior, 11th edn. Pearson, Boston, Massachusetts.
- Chase, D.L., Pepper, J.S. and Koelle, M.R. 2004. Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. Nature Neurosci. 7: 1096–1103.
- Claassen, D.E. and Kammer, A.E. 1986. Effects of octopamine, dopamine, and serotonin on production of flight motor output by thoracic ganglia of *Manduca sexta*. Dev. Neurobiol. 17: 1-14.
- Cook, C.N., Brent, C.S. and Breed, M.D. 2017. Octopamine and tyramine modulate the thermoregulatory fanning response in honey bees (*Apis mellifera*). J. Exp. Biol. 220: 1925-1930.
- Dickinson, M.H., Lehmann, F.O. and Chan, W.P. 1998. The control of mechanical power in insect flight. Amer. Zool. 38:718-728.
- Dombroski, T., Simoes, Z.L. and Bitondi, M.M. 2003. Dietary dopamine causes ovary activation in queenless *Apis mellifera* workers. Apidologie 34:281–289.
- Dominguez, J.M. and Hull, E.M. 2005. Dopamine, the medial preoptic area, and male sexual behavior. Physiol. Behav. 86:356–368.
- Draper, I., Kurshan, P.T., McBride, E., Jackson, F.R. and Kopin, A.S. 2007. Locomotor activity is regulated by D2-like receptors in *Drosophila*: an anatomic and functional analysis. Dev. Neurobiol. 67: 378–393.

- Evans, P.D. 1980. Biogenic amines in the insect nervous system. *Adv. Insect. Physiol.* 15: 317-473.
- Evans, P.D. 1986. Biogenic amine receptors and their mode of action. In: *Insect Neurochemistry and Neurophysiology*. Humana Press, pp. 117- 141.
- Fluri, P., Sabatini, A.G., Vecchi, M.A. and Wille, H. 1981. Blood juvenile hormone, protein and vitellogenin titres in laying and non-laying queen honeybees. *J. Apic. Res.* 20: 221–225.
- Fussnecker, B.L., Smith, B.H. and Mustard, J.A. 2006. Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey bee (*Apis mellifera*). *J. Insect Physiol.* 52: 1083-1092.
- Gilley, D.C. 2001. The behavior of honey bees (*Apis mellifera ligustica*) during queen duels. *Ethology* 107:601–622.
- Gilley, D.C. and Tarpy, D.R. 2005. Three mechanisms of queen elimination in swarming honey bee colonies. *Apidologie* 36:461–474.
- Gmeinbauer, R. and Crailsheim, K. 1993. Glucose utilization during flight of honeybee (*Apis mellifera*) workers, drones and queens. *J. Insect Physiol.* 39: 959-967.
- Gole, J.W., Orr, G.L. and Downer, R.G. 1987. Pharmacology of octopamine, dopamine, and 5-hydroxytryptamine-stimulated cyclic amp accumulation in the corpus cardiacum of the american cockroach, *Periplaneta americana* L. *Arch. Insect Biochem. Physiol.* 5:119–128.

- Grohmann, L., Blenau, W., Erber, J., Ebert, P.R., Strünker, T. and Baumann, A. 2003. Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. J. Neurochem. 86: 725-735.
- Hasselmo, M.E. 1995 Neuromodulation and cortical function: modeling the physiological basis of behavior. Behav. Brain Res. 67: 1-27.
- Harano, K., Sasaki, K. and Nagao, T. 2005. Depression of brain dopamine and its metabolite after mating in European honeybee (*Apis mellifera*) queens. Naturwissenschaften 92:310–313.
- Harano, K., Sasaki, M., Nagao, T. and Sasaki, K. 2008. Dopamine influences locomotor activity in honeybee queens: implications for a behavioural change after mating. Physiol. Entomol. 33:395–399.
- Harano, K., Sasaki, M. and Sasaki, K. 2007. Effects of reproductive state on rhythmicity, locomotor activity and body weight in European honeybee, *Apis mellifera* (Hymenoptera, Apini) queens. Sociobiology 50: 189-200.
- Harano, K. and Obara, Y. 2004. The role of chemical and acoustical stimuli in selective queen cell destruction by virgin queens of the honeybee *Apis mellifera* (Hymenoptera : Apidae). Appl. Entomol. Zool. 39:611–616.
- Harris, J.W. and Woodring, J. 1992. Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. J. Insect Physiol. 38: 29-35.
- Harvey, J.A. 1996. Serotonergic regulation of associative learning. Behav. Brain Res. 73: 47-50.

- Higginson, A.D., Barnard, C.J., Tofilski, A., Medina, L. and Ratnieks, F. 2011. Experimental wing damage affects foraging effort and foraging distance in honeybees *Apis mellifera*. *Psyche: A Journal of Entomology* 2011.
- Huber, R., Orzeszyna, M., Pokorny, N. and Kravitz, E.A. 1997. Biogenic amines and aggression: experimental approaches in crustaceans. *Brain Behav. Evol.* 1: 60-68.
- Humphries, M.A., Mustard, J.A., Hunter, S.J., Mercer, A., Ward, V. and Ebert, P.R. 2003. Invertebrate D2 type dopamine receptor exhibits age-based plasticity of expression in the mushroom bodies of the honeybee brain. *J. Neurobiol.* 55: 315-330.
- Hunt, G.J. 2007. Flight and fight: a comparative view of the neurophysiology and genetics of honey bee defensive behavior. *J. Insect Physiol.* 53:399–410.
- Ichikawa, N. and Sasaki, M. 2003. Importance of social stimuli for the development of learning capability in honeybees. *Appl. Entomol. Zool.* 38:203–209.
- Johnson, B.R. 2010. Division of labor in honeybees: form, function, and proximate mechanisms. *Behav. Ecol. Sociobiol.* 64:305–316.
- Kramer, P.F., Christensen, C.H., Hazelwood, L.A., Dobi, A., Bock, R., Sibley, D.R., Mateo, Y. and Alvarez, V.A., 2011. Dopamine D2 receptor overexpression alters behavior and physiology in *Drd2-EGFP* mice. *J. Neurosci.* 31:126–132.
- Laidlaw, H.H. and Page, R.E. 1997. Queen rearing and bee breeding. Wicwas Press, Cheshire, CT.
- Lange, A.B. 2009. Tyramine: from octopamine precursor to neuroactive chemical in insects. *Gen. Comp. Endocrinol.* 162:18–26.

- Lent, C.M. 1985. Serotonergic modulation of the feeding behavior of the medicinal leech. *Brain Res. Bull.* 14: 643-655.
- Lehmann, F.O. and Bartussek, J. 2017. Neural control and precision of flight muscle activation in *Drosophila*. *J. Comp. Physiol. A* 203:1-14.
- Lima, S.Q. and Miesenböck, G. 2005. Remote control of behavior through genetically targeted photostimulation of neurons. *Cell* 121:141-152.
- Linn, C.E., Poole, K.R. and Roelofs, W.L. 1994. Studies on biogenic amines and metabolites in nervous tissue and hemolymph of male cabbage looper moths—III. Fate of injected octopamine, 5-hydroxytryptamine and dopamine. *Comp. Biochem. Physiol.* 108:99-106.
- Linn, C.E. and Roelofs, W.L. 1986. Modulatory effects of octopamine and serotonin on male sensitivity and periodicity of response to sex pheromone in the cabbage looper moth, *Trichoplusia ni*. *Arch. Insect Biochem. Physiol.* 3: 161-171.
- Long, T.F. and Murdock, L.L. 1983. Stimulation of blowfly feeding behavior by octopaminergic drugs. *Proc. Natl. Acad. Sci. USA* 80: 4159-4163.
- McGraw, L.A., Clark, A.G. and Wolfner, M.F. 2008. Post-mating gene expression profiles of female *Drosophila melanogaster* in response to time and to four male accessory gland proteins. *Genetics* 179: 1395–1408.
- Mercer, A.R., Mobbs, P.G., Davenport, A.P. and Evans, P.D. 1983. Biogenic amines in the brain of the honey bee, *Apis mellifera*. *Cell Tissue Res.* 234: 655-677.

- Mezawa, R., Akasaka, S., Nagao, T. and Sasaki, K. 2013. Neuroendocrine mechanisms underlying regulation of mating flight behaviors in male honey bees (*Apis mellifera* L.). Gen. Comp. Endocrinol. 186:108–115.
- Mustard, J.A., Beggs, K.T. and Mercer, A.R. 2005. Molecular biology of invertebrate dopamine receptors. Arch. Insect Biochem. Physiol. 59: 103-117.
- Mustard, J.A., Blenau, W., Hamilton, I.S., Ward, V.K., Ebert, P.R. and Mercer, A.R. 2003. Analysis of two D1-like dopamine receptors from the honey bee *Apis mellifera* reveals agonist-independent activity. Mol. Brain Res. 113: 67-77.
- Mustard, J.A., Pham, P.M. and Smith, B.H. 2010. Modulation of motor behavior by dopamine and the D1-like dopamine receptor AmDOP2 in the honey bee. J. Insect Physiol. 56:422–430.
- Obara, Y., Fukano, Y., Watanabe, K., Ozawa, G. and Sasaki, K. 2011. Serotonin-induced mate rejection in the female cabbage butterfly, *Pieris rapae crucivora*. Naturwissenschaften 98: 989–993.
- Patki, G., Atrooz, F., Alkadhi, I., Solanki, N. and Salim, S., 2015. High aggression in rats is associated with elevated stress, anxiety-like behavior, and altered catecholamine content in the brain. Neurosci. Lett. 584:308–13.
- Pflugfelder, J. and Koeniger, N. 2003. Fight between virgin queens (*Apis mellifera*) is initiated by contact to the dorsal abdominal surface. Apidologie 34:249–256.
- Rillich, J. and Stevenson, P.A. 2014. A fighter's comeback: dopamine is necessary for recovery of aggression after social defeat in crickets. Horm. Behav. 66:696–704.

- Rillich, J. and Stevenson, P.A. 2015. Releasing stimuli and aggression in crickets: octopamine promotes escalation and maintenance but not initiation. *Front. Behav. Neurosci.* 9:95.
- Rhodes, J.S., Gammie, S.C. and Garland, T. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. *Integr. Comp. Biol.* 45: 438 – 455.
- Robinson, G.E. and Vargo, E.L. 1997. Juvenile hormone in adult eusocial Hymenoptera: gonadotropin and behavioral pacemaker. *Arch. Insect Biochem. Physiol.* 35: 559-583.
- Roeder, T. 2005. Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50: 447-477.
- Roeder, T., Seifert, M., Kähler, C. and Gewecke, M. 2003. Tyramine and octopamine: Antagonistic modulators of behavior and metabolism. *Arch. Insect Biochem. Physiol.* 54: 1-13.
- Santoro, D., Hartley, S., Suckling, D.M. and Lester, P.J. 2015. The stinging response of the common wasp (*Vespula vulgaris*): plasticity and variation in individual aggressiveness. *Insect. Soc.* 62:455–463.
- Sasaki, K., Matsuyama, S., Harano, K. and Nagao, T. 2012. Caste differences in dopamine-related substances and dopamine supply in the brains of honeybees (*Apis mellifera* L.). *Gen. Comp. Endocrinol.* 178:46–53.
- Sasaki, K. and Nagao, T. 2001. Distribution and levels of dopamine and its metabolites in brains of reproductive workers in honeybees. *J. Insect Physiol.* 47:1205–1216.

- Sasaki, K. and Nagao, T. 2013. Juvenile hormone-dopamine systems for the promotion of flight activity in males of the large carpenter bee *Xylocopa appendiculata*. *Naturwissenschaften* 100: 1183–1186.
- Saveer, A.M., Kromann, S.H., Birgersson, G., Bengtsson, M., Lindblom, T., Balkenius, A., Hansson, B.S., Witzgall, P., Becher, P.G. and Ignell, R., 2012. Floral to green: mating switches moth olfactory coding and preference. *Proc. Biol. Sci.* 279: 2314–22.
- Scheiner, R., Entler, B.V., Barron, A.B., Scholl, C. and Thamm, M. 2017. The Effects of Fat Body Tyramine Level on Gustatory Responsiveness of Honeybees (*Apis mellifera*) Differ between Behavioral Castes. *Front. Syst. Neurosci.* 11:55.
- Schneider, S.S. and DeGrandi-Hoffman, G. 2008. Queen replacement in African and European honey bee colonies with and without afterswarms. *Insect. Soc.* 55:79–85.
- Schneider, S.S., Painter-Kurt, S. and DeGrandi-Hoffman, G. 2001. The role of the vibration signal during queen competition in colonies of the honeybee, *Apis mellifera*. *Anim. Behav.* 61:1173–1180.
- Schrempf, A., Heinze, J. and Cremer, S. 2005. Sexual cooperation: Mating increases longevity in ant queens. *Curr. Biol.* 15: 267–270.
- Schulz, D.J. and Robinson, G.E. 1999. Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *J. Comp. Physiol. A* 184: 481–488.
- Schulz, D.J. and Robinson, G.E. 2001. Octopamine influences division of labor in honey bee colonies. *J. Comp. Physiol. A* 187: 53–61.



- Schulz, D.J., Sullivan, J.P. and Robinson, G.E. 2002. Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies. *Horm. Behav.* 42: 222-231.
- Summers, C.H. and Greenberg, N. 1995. Activation of central biogenic amines following aggressive interactions in male lizards, *Anolis carolinensis*. *Brain Behav. Evol.* 45: 339-349.
- Szczuka, A., Korczyńska, J., Wnuk, A., Symonowicz, B., Szwacka, A.G., Mazurkiewicz, P., Kostowski, W. and Godzińska, E.J., 2013. The effects of serotonin, dopamine, octopamine and tyramine on behavior of workers of the ant *Formica polyctena* during dyadic aggression tests. *Acta Neurobiol. Exp.* 73:495–520.
- Tanaka, E.D. and Hartfelder, K. 2004. The initial stages of oogenesis and their relation to differential fertility in the honey bee (*Apis mellifera*) castes. *Arthropod Struct. Dev.* 33: 431-442.
- Tarpy, D.R. and Fletcher, D.J. 1998. Effects of relatedness on queen competition within honey bee colonies. *Anim. Behav.* 55:537–543.
- Tarpy, D.R., Gilley, D.C. and Seeley, T.D. 2004. Levels of selection in a social insect: a review of conflict and cooperation during honey bee (*Apis mellifera*) queen replacement. *Behav. Ecol. Sociobiol.* 55:513–523.
- Tarpy, D.R., Hatch, S. and Fletcher, D.J. 2000. The influence of queen age and quality during queen replacement in honeybee colonies. *Anim. Behav.* 59:97–101.
- Tarpy, D.R. and Mayer, M.K. 2009. The effects of size and reproductive quality on the outcomes of duels between honey bee queens (*Apis mellifera* L.). *Ethol. Ecol. Evol.* 21:147–153.

- Wagener-Hulme, C., Kuehn, J.C., Schulz, D.J. and Robinson, G.E. 1999. Biogenic amines and division of labor in honey bee colonies. *J. Comp. Physiol. A* 184: 471–479.
- Wegener, J., Huang, Z.Y., Lorenz, M.W., Lorenz, J.I. and Bienefeld, K. 2013. New insights into the roles of juvenile hormone and ecdysteroids in honey bee reproduction. *J. Insect Physiol.* 59: 655-61.
- Winston, M.L. 1987. The biology of the honey bee. Harvard University Press, Cambridge, Massachusetts.
- Zmarlicki, C. and Morse, R.A. 1963. Drone congregation areas. *J. Apic. Res.* 2:64–66.