



HOKKAIDO UNIVERSITY

Title	Neuroethological studies of the patch use behavior in socially foraging chicks
Author(s)	辛, 秋红
Degree Grantor	北海道大学
Degree Name	博士(生命科学)
Dissertation Number	甲第12730号
Issue Date	2017-03-23
DOI	https://doi.org/10.14943/doctoral.k12730
Doc URL	https://hdl.handle.net/2115/68575
Type	doctoral thesis
File Information	XIN_QIUHONG.pdf



**Neuroethological studies of the patch use behavior
in socially foraging chicks**

**(社会採餌するニワトリ雛のパッチ利用行動に関
する神経行動学研究)**

A DISSERTATION

submitted to the Graduate School of Life Science,

Hokkaido University

in partial fulfillment of the requirements for the degree of

DOCTOR OF LIFE SCIENCE

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March 2017

CONTENTS

GENERAL INTRODUCTION	<i>Neuroethological studies of the patch use behavior in social foraging</i>	1
CHAPTER I	<i>Behavioral study: patch use behavior of socially foraging chicks</i>	7
1.1	Introduction	8
1.2	Materials and methods	10
1.3	Results	14
1.4	Discussion	16
1.5	Figures	18
1.6	Appendix	20
CHAPTER II	<i>Localized brain lesion study: selective contribution of the telencephalic arcopallium to the social facilitation of foraging efforts in domestic chicks</i>	25
2.1	Introduction	26
2.2	Materials and methods	30
2.3	Results	41
2.4	Discussion	45
2.5	Figures	51
CHAPTER III	<i>Neuroanatomical tract tracing study: distinct projection pattern of the lateral and medial arcopallium</i>	57
3.1	Introduction	58
3.2	Materials and methods	60
3.3	Results	64
3.4	Discussion	67
3.5	Abbreviation	69
3.6	Figures	72
GENERAL DISCUSSION	<i>Is the avian arcopallium analogous to the prefrontal cortex or amygdala in mammals?</i>	77

ACKNOWLEDGEMENTS	80
REFERENCES.....	82
LIST OF PUBLICATIONS.....	94
LIST OF OTHER PUBLICATION	95
SUPPLEMENTARY MATERIALS.....	98

GENERAL INTRODUCTION

*Neuroethological studies of the patch use behavior in
social foraging*

Behavioral ecology of social foraging

Classical foraging theory focuses on the perspective of a single forager (Charnov, 1976). Social interference by conspecifics in group foraging animals has been largely ignored. In more recent perspectives, however, the social foraging theory suggests that the individual decision-making is subject not only to food resources but also to conspecific behavior, which influences the consequence of individual foraging strategy (Giraldeau and Caraco, 2000). For example, foraging tactics often differentiate among individuals of foraging groups. When the food items are sharable, kleptoparasitism gives rise to two beneficial tactics, namely producer and scrounger, therefore comprising the producer-scrounger game (Giraldeau and Caraco, 2000). Here, producers forage themselves and scroungers exploit the finding by the producers. When foraging singly, animals take only the producer tactics. However, when accompanied by conspecifics that forage as producers, scrounger tactics may be more beneficial. Due to frequency dependence of these tactics, proportion of the scrounging individuals in foraging groups reaches a stable equilibrium, at which both tactics yield the same benefit.

Patch use behavior and prefrontal cortex

It is also to be considered that animals live in such environments that resources are distributed in patches. The foraging animals must leave a patch of food at a certain point of time before its resource is exhausted, as illustrated by the patch-use model by Charnov *et al.* (1976). According to the marginal value theorem adopted widely in behavioral ecology, optimal animals leave the patch when instantaneous gain rate declines to the level of average gain rate in the environment (Stephens & Krebs, 1986; Charnov *et al.*, 1976). A variety of

animals ranging from insects (Kasuya, 1982), birds (Kacelnik, 1984), to monkeys (Agetsuma, 1999) as well as humans (Smith & Winterhalder, 1992) were found to follow the marginal value theorem, implying that a common neuronal mechanism may underlies. However, so far, studies have not been done on the neural mechanism except few cases. Neurons in the dorsal anterior cingulate cortex of Macaca monkeys are reported to encode a decision variable that signals the relative value of leaving a depleting resource for a new one (Hayden *et al.*, 2011).

In these studies, the subject animals made choices in single foraging condition, which is different from the natural situation of social patch use behaviors in some aspects. In particular, the classical patch model can hardly tackle with the frequency dependent payoffs commonly associated with the social foraging (Giraldeau & Caraco, 2000). Actually, as noted above, socially foraging animals do not optimize foraging, because both of producers and scroungers gain equal benefit. Instead, frequency dependent nature of the producer-scrounger game leads to a stable equilibrium of a sub-optimal condition. How can animals modify their patch use behavior under social interference? What neural mechanisms could underlie the modification away from the individual optimization? Involvement of higher brain centers such as prefrontal cortex has been assumed to play a role.

Neural substrates of social foraging

In mammals, a number of cortical and subcortical structures have been implicated in foraging behavior (Floresco *et al.*, 2008; Wallis & Rushworth, 2014). These include the frontal cortex, specifically the anterior cingulate cortex and orbitofrontal cortex (Walton *et*

al., 2002, 2003, 2009; Schweimer *et al.*, 2005; Rudebeck *et al.*, 2006), and the basolateral amygdala (Floresco & Ghods-Sharifi, 2007). In addition, involvement of the basal ganglia, specifically the nucleus accumbens (Salamone *et al.*, 1994; Cousins *et al.*, 1996; Hauber & Sommer, 2009; but see Walton *et al.*, 2009) has been assumed. It should be noted that these brain regions are also responsible for social modulation. For example, orbitofrontal cortex damage impairs social cognition in the case of patient M.R. (Gazzaniga *et al.*, 2004). Recent imaging studies have revealed that the anterior cingulate cortex is active when human subject infer the evaluation of rewards generated by another individual (Apps & Ramnani, 2014). The basolateral amygdala was found modulate anxiety in the social interaction test (Gonzalez *et al.* 1996; Sajdyk & Shekhar, 1997). However, the foraging and the social interactions have been studied separately. Neural basis of social foraging behaviors have not been addressed both in humans and animals. The distinct evolutionary backgrounds in different taxa (particular birds and mammals) attracts a particular scientific interest.

Evolution of the avian and mammalian brains

Birds and mammals descend from a common stem amniote. Due to ~300 million years of separated evolution (Cambell & Reece, 2002), the avian brain structures are now quite distinct from those of mammals. Despite the distinct phylogeny, the apparent correspondence appears between the mammalian and avian brain regions (Jarvis *et al.* 2005). Several hypotheses have been proposed, in which one-to-one homologies between specific avian and mammalian pallial subdivisions have been claimed, such as the nuclear-to-layered hypothesis and the nuclear-to-claustrum/amygdala nuclei hypothesis (Jarvis *et al.* 2005). The issues of

the homological relationship of the mammalian and avian pallium remain highly controversial.

The use of domestic chick as a model

Despite brain structures distinct from mammals, domesticated fowl chicks (*Gallus gallus domesticus*) are useful for studying the social foraging and the underlying neural mechanisms. First, chicks forage in highly social circumstances (Nicol, 2015). Second, when exposed to a conspicuous moving object for several hours, newly hatched chicks as precocial birds selectively form a social attachment to that object, which is well known as filial imprinting (Matsushima et al., 2003). The intermediate medial mesopallium (IMM) was found to be evolved in the imprinting, especially for the acquisition process. Third, foraging decisions are socially modulated by conspecific individuals. Choice impulsiveness in chicks can be conditionally enhanced by competitive training experiences (Amita *et al.*, 2010), and this enhancement might involve the suppression of striatal neuronal activities, as elicited by the presence of the accompanying forager (Amita & Matsushima, 2014). Furthermore, foraging effort (Ogura & Matsushima, 2011) and operant peck latency (Amita & Matsushima, 2011) are socially facilitated in a reversible and contextual manner. Localized lesion to the tegmentum around the substantia nigra (SN, the major ascending dopaminergic nucleus) partially reduced the facilitation. However, selective depletion of dopaminergic neurons failed to reproduce the effect, and the facilitation remained unchanged (Ogura *et al.*, 2015). The underlying neural mechanisms are still unclear. In this thesis, with these behavioral and neural studies as backgrounds, I will introduce my studies using chicks as subjects.

Goal of the present study

My goal has been to elucidate the neural substrate of patch use behavior in social foraging condition in chicks. In chapter I, I will show how the patch use behavior is socially modulated. In chapter II, I show the neural substrates responsible for one of the social modulation, or social facilitation of running distance, in a series of localized brain lesion experiments. In chapter III, based on the results of lesion studies, I show neuroanatomical tract tracing study. I will argue that the social facilitation, but not other aspects of social modulation of foraging behavior, is mediated by efferent pathways from arcopallium of the avian telencephalon. Finally, based on these studies, I discuss possible analogous correspondence between the avian arcopallium and the mammalian prefrontal cortex or the amygdala.

CHAPTER I

***Behavioral study: patch use behavior of socially foraging
chicks***

1.1 Introduction

In neuroeconomics, tasks have been developed to mimic the ecological situations that animals face in wild, namely foraging under uncertain and competitive environments (Hayden *et al.*, 2011; Mobbs *et al.*, 2013). In these tasks, subjects are often required to select one of two targets via a directed action (such as an eye saccade or button-press). In nature, on the other hand, foragers encounter food sequentially and select one action out of several possibilities (Stephens & Krebs, 1986). For example, study of patch use behavior suggested that optimal animals must choose to leave a patch of food at a certain point of time before its resource is exhausted (Charnov *et al.*, 1976). It is therefore important to understand how animals select actions when foraging among food patches.

When groups of animals forage among food patches, an ideal free distribution may emerge due to interference among individuals (Fretwell & Calver, 1969; Křivan *et al.*, 2008). In the equilibrated condition, the number of competitive foragers in each patch will match the food rate available there. In simulated groups of foragers with a considerable interference, matching actually arises as an adaptive trait through selection by genetic algorithm (Seth, 2002). At the level of individual behavior, on the other hand, matching may inevitably arise from leaky integration of reward signals (Sugrue *et al.*, 2004) or as a by-product of actor–critic learning algorithm (Sakai & Fukai, 2008) in a manner irrelevant to the social foraging. It is therefore critical to examine whether matching is sensitive to the interference in group-foraging animals. More specifically, if and how animals modify the matching under social interference over food resource.

I addressed this issue by using chicks as subjects, because they forage in highly social

circumstances (for a recent review see Nicol, 2015). Chicks develop choice impulsiveness if trained in a group under fictitiously competitive conditions (Amita *et al.*, 2009; Mizuyama *et al.*, 2016). Foraging effort (running and pecking) is also socially facilitated in pairs (Ogura & Matsushima, 2011; Ogura *et al.*, 2015). Here, I examined how chicks behave among two patches of different food delivery rate. Particular attention was paid to whether reversal of the feeding rate changes the patch use, and whether the fictitious interference of food interacts with the changes.

1.2 Materials and methods

Subjects

Male domestic chicks (*Gallus gallus domesticus*) were purchased on post-hatch day 1 from a local supplier (Iwamura Poultry Ltd. /Hokkaido Central Poultry Ltd., Yubari, Japan). Chicks were communally housed in transparent plastic cages (15 cm × 28 cm, 12 cm high; kept at ca. 30°C) with a 12:12-h light:dark cycle with lights on at 08:00. Chicks received grains of millet and chick mash, with the amount adjusted daily such that body weight increased by ~5% per day, and that the chicks actively consumed food in tests. Water was freely available.

Ethical Note

I did not perform any invasive treatments or stressful handling during the experiments. When chick emitted distress calls in the experimental apparatus, I immediately stopped the experiment. I discarded 4 out of 64 chicks, and the present results were based on the remaining 60 chicks. The experiments were conducted under the guidelines and approval (#11-0042) of the Committee on Animal Experiments of Hokkaido University. The guidelines are based on the national regulations for animal welfare in Japan (Law for Humane Treatment and Management of Animals; after a partial amendment No. 68, 2005). After the experiments, chicks were euthanized using carbon dioxide.

Apparatus

An I-shaped maze was partitioned such that it had two lanes (12 × 88 × 40 cm, **Figure**

I-1B; Ogura & Matsushima, 2011). The lanes were separated via a transparent Plexiglas or mirror partition, and each lane accommodated a single chick except the habituation. Each of the terminal walls (one red and one yellow) held a pair of food trays (a patch), with one tray in each lane. I used Micro-robot (Mindstorms RCX, LEGO, Denmark) to deliver millet grains simultaneously to each of the paired trays. Although no actual food interference occurred, the chicks in the partitioned lanes experienced fictitious interference over the delivered food. Food delivery in the opposite patches was not linked, but independently controlled. Four 60-W light bulbs illuminated the maze, and chicks were video-recorded via a camera on the ceiling (DCR-SR65, Sony, Japan). This provided an aerial view of the running trajectories, which were traced offline using Move-Tr/2D 7.0 (Library Co., Japan).

Behavioral procedures

Chicks were habituated to the maze in daily sessions on post-hatch day 6 to day 7 (D6-7) (**Figure I-1A**). A pair of chicks was introduced to a lane with starter food (180 grains). After the food was consumed, the feeders were turned on to deliver millet according to the variable interval (VI) schedule. The delivery interval varied uniformly in 10–20 s with mean = 15 s, therefore VI15. Two grains of millet were delivered simultaneously, and the paired chicks received a total of 240 grains in ~16 min. After the delivered food was consumed, the chicks remained in the maze for an additional 2-min ‘no food (after feeding)’ period.

On D8–11, chicks were randomly allocated to one of three groups (**Figure I-1B**); ‘single’ (n=18), ‘paired’ (n=24), and ‘mirror’ (n=18). In the single group, chicks were individually tested in one lane. In the paired group, paired chicks were tested in the partitioned lanes. Chicks equally gained 1 grain per chick per delivery, and actual interference did not occur.

In the mirror group, chicks were individually tested in one lane, and the Plexiglas partition was replaced with a mirror. Biased food delivery started as soon as the chicks were introduced to the maze. One feeder (e.g. yellow) supplied grains on a VI10 schedule (range = 6.7–13.3 s, mean = 10 s) and the other (red) supplied grains on a VI30 schedule (range = 20–40 s, mean = 30 s). Color allocation was randomized among individuals. After the food delivery period, chicks were left in the maze for an additional 2-min ‘no food (after feeding)’ period. On D12–15, the feeding rate schedules were reversed between the two feeders. Behavior was also recorded during a 2-min ‘no food (before feeding)’ period.

Statistical analysis of data

I obtained the running distances and patch use ratios from the recorded trajectories as response variables. Running distance was the cumulative distance the subject run during the 16-min feeding period. Patch use ratio was the proportion of the time during which the subject stayed close (< 10 cm) to the VI10 feeder on D8–11, divided by the total stay time at both feeders. Group was a between-individual variable, which denotes foraging condition in the I-shaped maze, i.e., single, paired and mirror. Day was a within-individual variable, which denotes the post-hatch day when the behaviors were recorded.

Inter-group comparisons were made by two-way analysis of variance (ANOVA) with repeated measures based on type III sums of squares. Degrees of freedom (DFs) were adjusted for non-sphericity using the Greenhouse–Geisser correction. Note that I did not perform any test for sphericity, as I tried to avoid multiple statistic tests. F scores and corresponding P values were calculated based on the adjusted DFs. Post-hoc pairwise comparisons were made after a Holm’s correction with the significance level set at $P = 0.05$.

Statistical calculations were performed by using R (version 3.1.3, Windows version) and “ANOVAKUN 4.8.0” add-on (programmed by Dr. Ryuta Iseki, <http://riseki.php.xdomain.jp/index.php?ANOVA%E5%90%9B>).

1.3 Results

During the feeding period, the chicks actively shuttled between the two patches. See **Figure I-1C** for representative trajectories on D11 (left) and D12 (right). A high degree of synchrony appeared among chicks in the paired group. After the reversal, chicks in all three groups quickly switched their patch use, staying at the VI10 patch longer than the alternative VI30 patch.

For running distance (**Figure I-2A**) on D8–11 (pre-reversal), I observed a significant main effect of group ($F_{2,57} = 11.36$, $P = 0.0001$), day ($F_{2.77,158.07} = 93.27$, $P < 0.0001$), and the interaction ($F_{5.55,158.07} = 9.01$, $P < 0.0001$). Multiple comparisons revealed significant difference between single vs. paired and single vs. mirror, but not between paired vs. mirror; see Appendix for detailed statistical data. On D12–15 (post-reversal), I observed a significant main effect of group ($F_{2,57} = 26.49$, $P < 0.0001$) and day ($F_{2.49,142.06} = 8.966$, $P = 0.0001$), but not the interaction ($F_{4.98,142.06} = 0.656$, $P = 0.6573$). Significant difference occurred between single vs. paired, single vs. mirror, but not between paired vs. mirror.

The patch use ratio on D8–11 also differed among the three groups of chicks (**Figure I-2B**). I observed a significant main effect of group ($F_{2,57} = 11.11$, $P = 0.0001$), but not day ($F_{2.31,131.45} = 2.665$, $P = 0.0654$) or interaction ($F_{4.61,131.45} = 1.016$, $P = 0.4076$). On D12–15, I found a significant effect of the interaction ($F_{5.6,159.55} = 3.243$, $P = 0.0060$), but not group ($F_{2,57} = 2.304$, $P = 0.1091$) or day ($F_{2.8,159.55} = 2.543$, $P = 0.0623$).

On D12 and afterwards, most chicks started to run as soon as they were introduced to the maze, even before food delivery was turned on. The patch use ratio during the ‘no food

(before feeding)’ period could thus represent the memorized value of the patches (**Figure I-2C**). Significant main effects occurred in group ($F_{2,55} = 4.160$, $P = 0.0208$), day ($F_{2,66,146.08} = 27.49$, $P < 0.0001$), and interaction ($F_{5,31,146.08} = 3.052$, $P = 0.0104$). On D12 and D13, I observed significant differences between paired vs. single and paired vs. mirror, but not between single vs. mirror (see Appendix); no significant inter-group differences appeared on D14 or D15.

Chicks continued running for an additional few minutes after the food delivery was turned off. The patch use ratio in the ‘no food (after feeding)’ period could thus represent an aftereffect based on short-term memory of the most recent food gain (**Figure I-2D**). On D8–11, I observed a significant main effect of group ($F_{2,57} = 6.008$, $P = 0.0043$) and day ($F_{2,74,156.19} = 3.625$, $P = 0.0173$), but no interaction ($F_{5,48,156.19} = 1.398$, $P = 0.2234$). On D12–15, a significant main effect occurred in day ($F_{2,26,128.93} = 4.466$, $P = 0.0103$), but not in group ($F_{2,57} = 0.2863$, $P = 0.7521$) or interaction ($F_{4,52,128.93} = 1.784$, $P = 0.1275$).

1.4 Discussion

The running distance data (**Figure I-2A**) confirmed previous findings regarding the social facilitation of foraging effort (Ogura & Matsushima, 2011). Although the effect was weaker, the mirror also facilitated running. Thus, perceived interference in group foraging was sufficient, even when it was caused by the reflection of the subject.

The paired chicks matched strictly to the feeding rate throughout D8–11 (**Figure I-2B**). The strict matching could be an optimal trait, as it would enable foraging chicks to minimize the risk of food gain reduction by disproportionately high level of competition to the gain rate available there. On the other hand, the facilitated running is not critical for the strict matching behavior, because chicks of the mirror group showed under-matching comparable to the single chicks. Synchronized foraging also does not account for this effect, because matching remained unchanged even when a localized lesion to arcopallium of telencephalon impaired the synchrony (Chapter II).

After the reversal on D12, chicks quickly switched their patch use ratio (**Figure I-1C**). However, on the patch use ratio on D12-15 (**Figure I-1C**), significant interaction occurred in the group $\times$ day. Most probably, the lasting memory effect (**Figure I-1C**, see below) counteracted the strict matching, shifting the patch use ratio slightly higher on D12-13.

On D13 particularly, the paired chicks stayed longer at the previously more profitable feeder (**Figure I-2C**), suggesting an improved long-term memory for the patch values. Such effect did not appear in the mirror chicks, which also showed facilitated running distance. On the other hand, the patch use time after feeding did not differ among the three groups (**Figure I-2D**). Short-term memory for the patch value could be formed similarly in all three

conditions. Taken together, we may assume two independent factors that control the patch-use behavior, i.e., lasting memory of the patch value, and quickly rewritable memory due to the recent gain.

The present results serve a clear empirical evidence for the modifiability of patch-use decisions by group-foraging, and are thus consistent with the Seth (2002)'s proposal that resource interference could cause matching to evolve (Seth, 2002). This, however, does not contradict with the idea of intrinsic matching mechanisms (Sakai & Fukai, 2008). It is noticed also that weak matching (under-matching) occurred also in the single and mirror condition (**Figure I-2B**). Matching could be a predisposed trait in Galliformes birds (Nicol, 2015), shaped through selection by group foraging.

The improved matching and patch memory would contribute to the collective intelligence, and group-foraging animals could gain a fitness advantage as has been illustrated in house sparrows (Belmaker *et al.*, 2012). The proximate mechanisms of matching behavior, or those neuro-cognitive processes involved in the appropriate selection of stay/leave actions, still remain largely unknown. A pharmacological study of patch use time (Matsunami *et al.*, 2012) revealed the involvement of serotonergic neuromodulation, suggesting a possible link with individual personality (Dingemanse & Réale, 2005). Further experimental characterization is necessary for the patch use in social foraging conditions, both in proximate and ultimate causes of behavior.

1.5 Figures

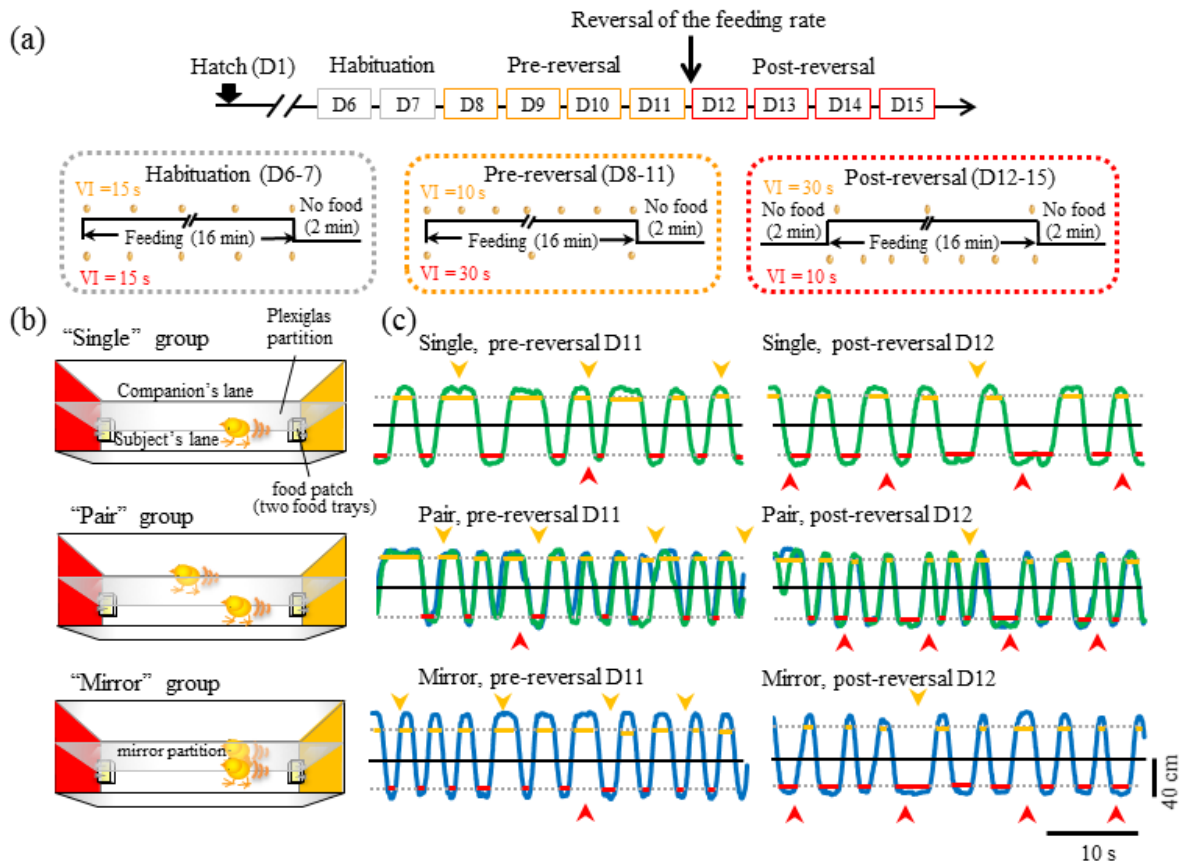


Figure I-1. (A) Procedures of behavioral tests on post-hatch days 6–15. (B) Schematic illustration of I-shaped maze with two lanes partitioned by a Plexiglas wall, and two terminal food patches on the yellow and the red wall, each composed of two food trays. Three groups of chicks were examined either in the ‘single’, ‘paired’, and ‘mirror’ conditions. (C) Representative trajectories. The y-axis indicates the position along the maze (Yellow: top and Red: bottom), and the x-axis is the time. Arrowheads denote the timing of food delivery, and horizontal rods indicate the stay time for each visit. Horizontal black lines indicate the midpoint of the maze.

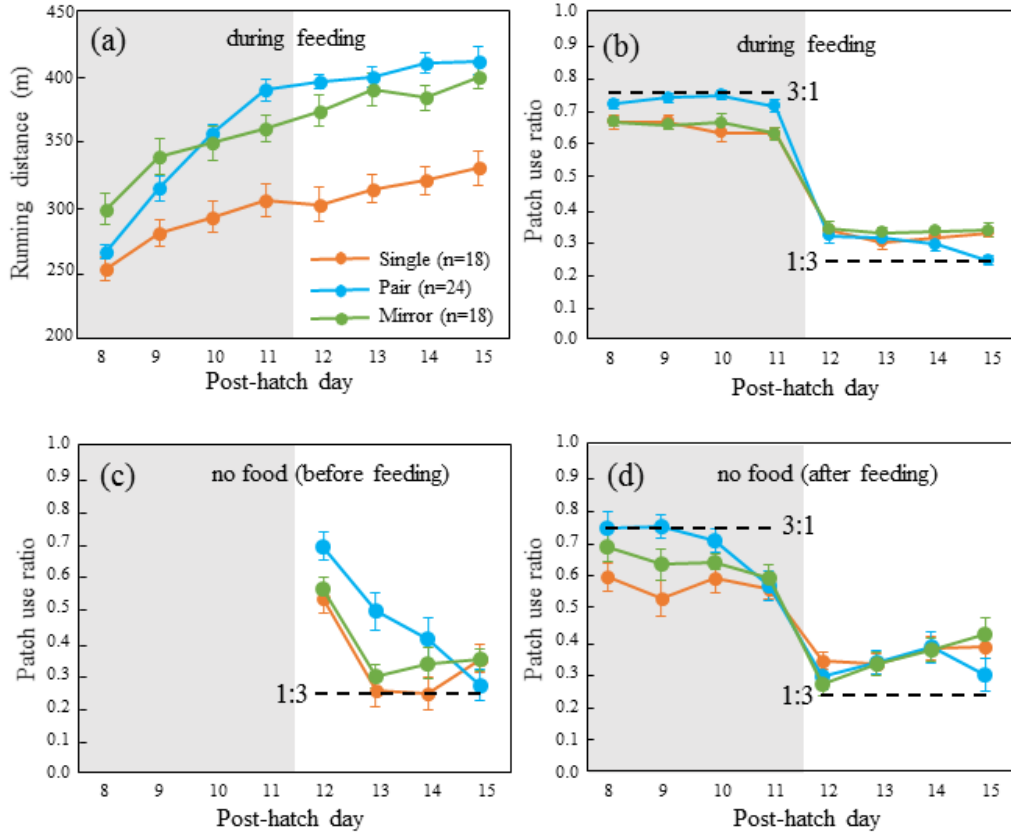


Figure I-2. (A) Averaged running distance (y-axis) plotted against the post-hatch day; 8–11 for the pre-reversal, and 12–15 for the post-reversal tests. (B–D) Patch use ratio at the more profitable feeder in the pre-reversal test (y-axis) plotted against the post-hatch day. Dashed horizontal lines indicate the level of 0.75 (3:1) and 0.25 (1:3). During the 16 min feeding period (B), the ‘no food (before feeding)’ period (C), and ‘no food (after feeding)’ period (D).

1.6 Appendix

Running distance during feeding (Figure I-2A)

Pre-reversal tests

Table 1.1.1: Two-way repeated measures ANOVA

Group	$F_{2,57} = 11.3572$	$P = 0.0001$
Day	$F_{2.77,158.07} = 93.2664$	$P = 0.0000$
Interaction (group : day)	$F_{5.55,158.07} = 9.0098$	$P = 0.0000$

Table 1.1.2: Post-hoc pairwise comparisons

Single : Paired	$t=4.1020$ $P=0.0003$
Single : Mirror	$t=4.2434$ $P=0.0002$
Paired : Mirror	$t=0.4344$ $P=0.6657$

Table 1.1.3: One-way ANOVA of running distance in each pre-reversal day

	Day 8		Day 9		Day 10		Day 11	
Single :								
Paired:	$F_{2,57}$	$P=0.0022$	$F_{2,57}$	$P=0.0031$	$F_{2,57}$	$P=0.0001$	$F_{2,57}$	$P=0.0000$
Mirror	$= 6.8287$		$= 6.4206$		$= 11.3023$		$= 18.1259$	

Table 1.1.4: Post-hoc pairwise comparisons of running distance in each pre-reversal day

	Day 8		Day 9		Day 10		Day 11	
Single: Paired	$t=1.1010$	$P=0.2755$	$t=2.2291$	$P=0.0595$	$t=4.4879$	$P=0.0001$	$t=5.9952$	$P=0.0000$
Single : Mirror	$t=3.5700$	$P=0.0022$	$t=3.5628$	$P=0.0022$	$t=3.7253$	$P=0.0009$	$t=3.6745$	$P=0.0011$
Paired : Mirror	$t=2.7155$	$P=0.0175$	$t=1.5796$	$P=0.1197$	$t=0.5055$	$P=0.6152$	$t=2.0670$	$P=0.0433$

Post-reversal tests

Table 1.2.1: Two-way repeated measures ANOVA

Group	$F_{2,57} = 26.4866$	$P=0.0000$
Day	$F_{2.49,142.06} = 8.9664$	$P=0.0001$
Interaction (group : day)	$F_{4.98,142.06} = 0.6555$	$P=0.6573$

Table 1.2.2: Post-hoc pairwise comparisons

Single : Paired	$t= 7.0552$	$P=0.0000$
Single : Mirror	$t=5.2823$	$P=0.0000$
Paired : Mirror	$t=1.4082$	$P=0.1645$

Patch use ratio during feeding (Figure I-2B)

Pre-reversal tests

Table 2.1.1: Two-way repeated measures ANOVA

Group	$F_{2,57} = 11.1134$	$P=0.0001$
Day	$F_{2.31,131.45} = 2.6651$	$P=0.0654$
Interaction (group : day)	$F_{4.61,131.45} = 1.0162$	$P=0.4076$

Table 2.1.2: Post-hoc pairwise comparisons

Single : Paired	$t=4.1281$	$P=0.000$
Single : Mirror	$t=0.2820$	$P=0.7789$
Paired : Mirror	$t=3.8266$	$P= 0.0006$

Post reversal tests

Table 2.2.1: Two-way repeated measures ANOVA

Group	$F_{2,57} = 2.3037$ $P = 0.1091$
Day	$F_{2.8,159.55} = 2.5430$ $P = 0.0623$
Interaction (group : day)	$F_{2.8,159.55} = 3.2434$ $P = 0.0060$

Table 2.2.2: One-way ANOVA in each post-reversal day

	Day 12		Day 13		Day 14		Day 15	
Single : Paired: Mirror	$F_{2,57}$ = 0.2795	$P = 0.7572$	$F_{2,57}$ = 0.5262	$P = 0.5937$	$F_{2,57}$ = 1.2569	$P = 0.2923$	$F_{2,57}$ = 10.2797	$P = 0.0002$

Table 2.2.3: Post-hoc pairwise comparisons in each post-reversal day

	Day 15	
Single : Paired	$t = 3.7028$	$P = 0.0010$
Single : Mirror	$t = 0.2326$	$P = 0.8169$
Paired : Mirror	$t = 3.9514$	$P = 0.0006$

Patch use ratio before feeding in the post-reversal tests (Figure I-2C)

Table 3.1: Two-way repeated measures ANOVA

Group	$F_{2,55} = 4.1599$ $P = 0.0208$
Day	$F_{2.66,146.08} = 27.4860$ $P = 0.0000$
Interaction (group : day)	$F_{5.31,146.08} = 3.0522$ $P = 0.0104$

Table 3.2: Post-hoc pairwise comparisons

Single : Paired	$t = 2.7925$ $P = 0.0215$
Single : Mirror	$t = 0.9125$ $P = 0.3655$
Paired : Mirror	$t = 1.8576$ $P = 0.1372$

Table 3.3: One-way ANOVA in each post-reversal day

	Day 12		Day 13		Day 14		Day 15	
Single :								
Paired:	$F_{2,55}$	$P=0.0080$	$F_{2,55}$	$P=0.0042$	$F_{2,55}$	$P=0.1895$	$F_{2,55}$	$P=0.3053$
Mirror	= 5.2799		= 6.0571		= 1.7144		= 1.2125	

Table 3.4: Post-hoc pairwise comparisons in each post-reversal day

	Day 12		Day 13	
Single : Paired	$t=2.9726$	$P=0.0131$	$t=3.1836$	$P=0.0072$
Single : Mirror	$t=0.5147$	$P=0.6088$	$t=0.5507$	$P=0.5841$
Paired : Mirror	$t=2.4681$	$P=0.0334$	$t=2.6439$	$P=0.0213$

Patch use ratio after feeding (Figure I-2D)*Pre-reversal tests*

Table 4.1.1: Two-way repeated measures ANOVA

Group	$F_{2,57} = 6.0075$	$P=0.0043$
Day	$F_{2,74,156.19} = 3.6249$	$P=0.0173$
Interaction (group : day)	$F_{5,48,156.19} = 1.3983$	$P=0.2234$

Table 4.1.2: Post-hoc pairwise comparisons

Single : Paired	$t= 3.4662$	$P= 0.0030$
Single : Mirror	$t= 1.8306$	$P= 0.1448$
Paired : Mirror	$t= 1.5092$	$P= 0.1448$

Post-reversal tests

Table 4.2.1: Two-way repeated measures ANOVA

Group	$F_{2,57} = 0.2863$	$P = 0.7521$
Day	$F_{2.62,128.93} = 4.4655$	$P = 0.0103$
Interaction (group : day)	$F_{4.52,128.93} = 1.7844$	$P = 0.1275$

CHAPTER II

Localized brain lesion study: selective contribution of the telencephalic arcopallium to the social facilitation of foraging efforts in domestic chicks

2.1 Introduction

To be adaptive, animals must decide how much cost to invest while foraging for food. In mammals, a number of cortical and subcortical structures have been implicated in foraging behavior (Floresco *et al.*, 2008; Wallis & Rushworth, 2014). Of these, the orbitofrontal cortex (OFC) is critical for processing the time that foragers must wait for food (delay-cost; Rudebeck *et al.*, 2006), whereas the anterior cingulate cortex (ACC) is critical for the amount of effort that foragers must invest to reach and consume food (effort-cost; Walton *et al.*, 2006). Experimental disconnection between the ACC and basolateral amygdala (BLA), as well as inactivation of BLA (Floresco & Ghods-Sharifi, 2007), causes an effort-cost aversion similar to that caused by an ACC lesion. Depending on the type of cost, different subsets of these circuits are recruited during decision-making such that the benefit-cost consequences are appropriate for the ecological and social needs.

Social context is critical when making decisions about effort cost investment. Although classical theories of foraging have assumed single foraging individuals (Charnov, 1976; Stephens & Krebs, 1986), recent behavioral studies clearly support the contribution of inter-individual interactions, such as kleptoparasitism in flocks of birds (Giraldeau & Dubois, 2008), and forms of social cognition such as empathy and perception of fairness in humans (Singer *et al.*, 2006). Additionally, recent imaging studies have revealed a “mentalizing” network in a region of the ACC that is active when an individual infers the subjective evaluation of rewards generated by another individual (Apps & Ramnani, 2014). However, exactly how social contexts (such as the presence or actions of others) influence foraging decisions remains unclear.

Despite brain structures distinct from mammals, domesticated fowl chicks (*Gallus gallus domesticus*) are useful for studying the social modulation of cost investments and the underlying neural mechanisms. First, chicks make choices depending on the delay-cost (Izawa *et al.*, 2003) and the effort cost (Aoki, Csillag *et al.*, 2006), and the relevant brain regions have been localized by lesion experiments. The NAc and surrounding areas in the medial striatum are involved in delay discounting (Izawa *et al.*, 2003), whereas the arcopallium (Arco) is associated with food peck effort (Aoki, Csillag *et al.*, 2006) in a doubly dissociated manner (Aoki, Suzuki *et al.*, 2006). Second, foraging decisions are socially modulated by conspecific individuals. Choice impulsiveness in chicks can be conditionally enhanced by competitive training experiences (Amita *et al.*, 2010), and this enhancement might involve the suppression of striatal neuronal activities, as elicited by the presence of the accompanying forager (Amita & Matsushima, 2014). Furthermore, foraging effort (Ogura & Matsushima, 2011) and operant peck latency (Amita & Matsushima, 2011) are socially facilitated in a reversible and contextual manner. Based on the “drive” theory (Zajonc, 1965), Ogura and her colleagues have initially hypothesized that ascending dopaminergic pathway is involved in the social facilitation. Actually, localized lesion to the tegmentum around the substantia nigra (SN, the major ascending dopaminergic nucleus) partially reduced the facilitation. However, selective depletion of dopaminergic neurons failed to reproduce the effect, and the facilitation remained unchanged (Ogura *et al.*, 2015). Because the lesioned area included fiber tracts descending from the telencephalon (occipito-mesencephalic tract, OM), they subsequently hypothesized that the Arco mediates the social facilitation through its descending projections to the tegmentum (Ogura *et al.* 2015).

Functional contributions of the Arco are yet only poorly understood. It is a region of the

avian pallium in the telencephalon, and was incorrectly labelled the archistriatum before the nomenclature reform (Reiner *et al.*, 2004). The Arco was thereafter considered to be a homologue of the mammalian amygdala because lesion to Arco reduced escape responses in adult ducks (Phillips, 1964). In accordance with this, electrical stimulation of the Arco caused freely-behaving chickens to emit crow-like vocalizations (Phillips *et al.*, 1972). Another dominant theory held that the Arco was part of the motor/premotor cortex (Veenman *et al.*, 1995), or more specifically, part of the pallial area involved in oro-facial motor control (Wild *et al.*, 1985). In songbirds, localized lesion of the robust nucleus of the Arco impaired song production (Nottebohm *et al.*, 1976). The Arco was also shown to code working memory for sound localization in barn owls (Knudsen & Knudsen, 1996a), suggesting that this region is similar to the lateral prefrontal cortex. Finally, Arco lesions in chicks led to avoidance of a costly option associated with a larger food reward (Aoki, Csillag *et al.*, 2006), suggesting a function analogous to the mammalian ACC or BLA.

In the present study, I examined whether and how the Arco functionally contributes to social facilitation. In addition to the foraging effort (running distance), I paid attention to the patch use ratio, which measures how long chick stays at feeders of different food delivery rate. As shown in Chapter I, I have found that a biased food delivery to two feeders caused a biased stay time, and that the paired chicks showed a significantly better matching to the delivery rate than the chicks in single condition. I assume that ratio of the stay time (patch use ratio) represents the relative value associated with the feeders (as of the cue period neuronal activities in Arco neurons; Aoki *et al.*, 2003). If the patch use ratio changed after the lesion, it is reasonable to argue that the effects on the foraging efforts could be indirectly caused by the altered value representations. In addition, I measured a behavioral index of

motor skill of pecking in some of the chicks tested.

I also examined the lesion effect of nearby structure, the nucleus taeniae of the amygdala (TnA). The TnA is supposed to be homologous to the amygdala (Cheng *et al.*, 1999), particularly to the sub-pallial medial amygdala of mammals (Yamamoto *et al.*, 2005). Furthermore, I examined nidopallium (Nido) located just dorsal to the Arco, because our Arco lesion often extended to Nido. The effect of the Arco lesion could thus be due to the collateral damages to Nido. It is also to be noted that, in pigeons, particularly its caudo-lateral part of Nido (NCL) is supposed to play an analogous function to prefrontal cortex in mammals (Diekamp, Kalt, *et al.*, 2002; Lissek & Güntürkün, 2003).

As a separate experiment, I examined the lesion effect on the operant peck latency. As described above (Amita & Matsushima, 2011), the peck latency is contextually shortened in the social (triplet) condition. If the shortening in peck latency changed after the lesion, we may argue that the effects on the foraging efforts were caused by a more general reason such as incapability to visually perceive conspecifics.

2.2 Materials and methods

Subjects and Ethical Note

I used male domestic chicks (*Gallus gallus domesticus*, White Leghorn strain). On post-hatch day 1 (presumed hatching day), I purchased new hatchlings from a local supplier (Iwamura poultry Ltd. Hokkaido establishment, Yubari, Hokkaido, Japan). Chicks were paired and communally housed in transparent plastic cages (15 cm × 28 cm × 12 cm) in a room illuminated according to a 12:12-h light:dark cycle with the light period starting at 08:00 a.m. The temperature of the room was kept constant at ca. 30 °C.

The chicks received two types of food, i.e., grains of millet and chick mash food. The total amount of food per day was adjusted so that (1) the body weight of the chicks gradually increased and (2) the chicks actively consumed food during experiments. Chicks were given mash food from post-hatch day 1. Specifically, they received 2 g (post-hatch days 1–3) and 2.5 g (day 4 and afterwards) of mash food. Grains of millet were added from post-hatch day 2. The amounts of grains were 2 g (days 2–3) and 2.5 g (day 4 and afterwards). Until post-hatch day 2, all chicks were communally fed. From post-hatch day 3, chicks were individually fed in cages that were visually separated by black boards. Water was freely available.

The experiments were conducted under the guidelines and approval of the Committee on Animal Experiments of Hokkaido University. The guidelines are based on the national regulations for animal welfare in Japan (Law for Humane Treatment and Management of Animals; after a partial amendment No. 68, 2005). After the experiments, the brains of the chicks were dissected under deep anaesthesia. In cases in which surgical operations were not

conducted, the chicks were euthanized with carbon dioxide.

Apparatus

I-shaped maze (Experiments 1 and 2)

I used the same I-shaped maze equipped with two lanes (each 12 cm wide, 88 cm long with 40 cm high side walls; **Figure II-1A**) as used in Chapter I. Briefly, the two lanes were separated by a transparent Plexiglas partition. Except noted otherwise, each lane accommodated one chick at a time. One terminal wall was painted red, and the other yellow (Experiment 1) or blue (Experiment 2). Each wall was equipped with a food patch comprising a pair of food trays, such that there was one tray at each end of a lane. Each tray was a rectangular plastic box (3 cm wide, 4 cm long and 2 cm deep) with a sponge on the bottom of the box. The two trays on each wall were placed side by side, but separated by the Plexiglas wall, so that each chick was individually fed without interference regarding the delivered food. A micro-robot (RCX, LEGO Mindstorms) delivered grains of millet simultaneously to both paired trays in a patch, such that the chicks could be fed concurrently, and thus experienced fictitious interference regarding the food. One micro-robot fed the yellow patch, and another fed the red patch independently. If a lane did not accommodate a chick, food was not delivered to that lane. To prevent the chicks from associating the mechanical sounds made by the micro-robots with the food reward, I positioned two dummy motors close to the maze. These generated the same motor sounds as the micro-robots at variable intervals (mean = 2.5 s, range = 1.5 to 3.5 s) as distractors. The entire apparatus was placed in a dark room maintained at ca. 25–30 °C and illuminated by four 60 W white light bulbs located above the maze.

I recorded the behavior of the chicks using a video camera/recorder (DCR-SR65, Sony, Japan; 30 frames per sec) placed on the ceiling above the I-maze, providing an aerial view of the subjects. Each chick was marked with a piece of fluorescent colored tape attached to the head. The trajectory of the foraging chicks was tracked offline using Move-Tr/2D 7.0 software (Library Co., Japan) on a PC.

Operant chamber (Experiment 3)

A hand-made operant box (20 cm × 20 cm × 19 cm, kept at ca. 27–30 °C) was used for recording behaviors (**Figure II-1C**). One of the surrounding walls was equipped with a liquid crystal display (LCD) monitor with the following specifications: size 10.4", 800 × 600 pixels; Logitech LCM-T102AS, Japan; flash rate: 56–75 Hz; brightness: 230 cd/m²; and pitch size: 0.264 × 0.264 mm. The LCD monitor was covered with a 1-mm thick transparent layer of Plexiglas for protection of the surface, and a frame-type optical touch sensor (size 10.4", E10D03U-30 without-glass, TouchTEK Co., Yokohama, Japan) for detecting chick pecking. The LCD monitor with the touch sensor was separated from the chick chamber by a 1-mm thick transparent Plexiglas plate. The plate had two open windows (each 2.5 cm × 3 cm, placed side by side at 3 cm, 4 cm above the floor level) through which the chicks pecked at visual stimuli (color cues) displayed on the LCD monitor at positions corresponding to the windows. Red or green rectangles appeared on the white screen as cue stimuli.

The presentation of visual cues on the monitor and the activity of the solenoids were coordinated, and I recorded the timing of pecking behavior using a programme written using LabView (ver.2010, National Instruments Co., Austin, Texas, USA). The operant box was

separated into two chambers by a transparent Plexiglas partition, one accommodating the subject chick and the other accommodating two companion chicks. The chick behavior was monitored using color CCD cameras (250 k pixels, MTV-54B(K)ON, Akizuki-Denshi Co., Tokyo, Japan) placed in the chamber.

Behavioral procedures

Running distance, synchrony, and patch use ratio in experiments 1 and 2

On post-hatch day 5 and 6, chicks were habituated to the maze in single daily sessions. As part of the habituation process, ca.100 grains of millet were placed at the midway point of the maze, and ca. 40 grains were placed in each of the trays on the sides such that chicks received about ca. 180 grains as starter food. A pair of cagemate chicks was then introduced into the lane with the starter food. Once the chicks had consumed the food, they received a brief intermission of about 1 min before the feeders started to deliver millet grains according to a VI15 schedule, i.e., at variable intervals with mean = 15 s (uniformly in a range of 10–20 s). Two grains of millet were delivered at a time, and the pair of chicks received a total of 120 grains (60 deliveries) from each patch during a ca. 16 min period. After the delivered food was consumed, the chicks were left in the maze for an additional 2 min.

After habituation, the chicks were tested for 4 days with two test sessions per day. I conducted four tests before and four tests after lesion (pre-lesion and post-lesion conditions, respectively). Each test comprised three consecutive phases: single #1, paired, and single #2 (**Figure II-1A**). The lesion operation was conducted on the day after the last pre-lesion tests, i.e., post-hatch day 9 or 11 (Experiment 1 or 2, respectively), and the chicks were allowed to recover for 1–2 days before the post-lesion tests.

In Experiment 1, asymmetric food delivery started as soon as the subject chicks were introduced to the maze (**Figure II-1B**). One feeder supplied food according to a VI10 schedule (mean = 10 s, range = 6.7–13.3 s) and the other had a VI30 schedule (mean = 30 s, range = 20–40 s), so that the former feeder was three times more profitable than the latter. Color assignment was pseudo-randomized among individual chicks. Chicks received two grains of millet at a time. One phase consisted of 45 food deliveries (90 grains) from the more profitable VI10 feeder, and 15 food deliveries (30 grains of millet) from the other VI30 feeder. The duration of each phase was approximately 8 min. In the single #1 phase, chicks were tested individually. After completion of the single #1 phase, a companion chick (cagemate) was introduced into the other lane of the maze, and the paired phase started immediately. During the paired phase, grains were simultaneously delivered to the chicks in both lanes. Finally, in the single #2 phase, the companion was removed from the maze, and the subject chick resumed foraging independently. After the food supply was terminated, the subject was left in the maze for an additional 2 min.

In order to check if the lesion impaired motor function, I examined the pecking food behavior as reported previously (Aoki, Csillag *et al.*, 2006). On the first day after the lesion, namely just before the first post-lesion test, single chick placed in the home cage was given a plastic box (4 cm x 6 cm, 2 cm deep) containing 6 grains of millet for four consecutive times with a brief intervals of 5 min. The grains were placed on a sponge on the floor of the box. I counted how many times the chick pecked until the 6 grains were all consumed, averaged the number and divided it by 6 to yield an index to denote the number of pecks per grain (κ ; Matsushima *et al.*, 2008).

As Experiment 2 was actually executed as a pilot experiment before Experiment 1, it had

a somewhat different schedule. First, during the acclimatization phase, the chicks consumed 20 grains supplied to each feeder (40 grains in total). Second, both of the terminal patches delivered food in a symmetric manner according to the same VI15 schedule (mean = 15 s, range = 10–20 s), and one grain of millet was delivered at a time. Third, one of the terminal walls was painted blue, rather than yellow. However, I noticed no effect of the blue compared with the yellow wall.

Response peck latency in experiment 3

Chicks were initially habituated to the chamber, and trained to peck the color cue in groups of three individuals. Thereafter, chicks were tested in two phases, i.e., single and triplet (**Figure II-1 C**). In the triplet phase, the subject was separated from two companion chicks by a Plexiglas partition, and received a controlled amount of food that was not scrounged by the companions. Red and green rectangles were displayed as cues. One of the cues was associated with a food reward (S+) and the other with no food (S-). Cue assignment was randomized among individuals. **Figure II-1 D** shows the procedure of a single trial. First, a color cue was presented for 2 s without any preceding stimuli. When the subject chick pecked at the S+ cue once or more, 6 grains of millet were delivered to the central food tray after a brief mechanical lag (0.29 s on average).

In order to examine the lesion effects on the response peck latency in those subjects after substantial training, chicks were trained in three phases: habituation, auto-shaping, and differential training. Habituation took place on post-hatch days 2–3, and auto-shaping took place on days 3–4. Both types of training were administered in one block per day. In the habituation phase, triplets of chicks were introduced to the chamber without the Plexiglas

partition for 20 min, and given 80 grains of millet without any cues. In the auto-shaping phase, chicks were trained to peck at a white plastic rod (5 mm in outer diameter) that was manually presented to the subjects in 40 trials. Two grains of millet was delivered as soon as one of the three chicks pecked at the rod.

Those chicks were tested to successfully associated the color cues with subsequent reward. To do that, on days 5–7, the chicks received two blocks of differential training per day, one block in the single phase and another in the triplet phase (**Figure II-1C**). The order of the two phases was counter-balanced among individuals. Each block consisted of 50 pseudo-randomly arranged trials including 20 trials with a rewarding color (S+ for 6 grains), 20 trials with a non-rewarding color (S- for no food), and 10 binary choice trials in which the chick had to choose between S+ and S-. In the binary trials, the chicks were not rewarded, regardless of whether they had correctly pecked at the S+. In the triplet phase, the companion chicks received two grains when the subject successfully pecked and received food in the S+ trials.

On days 8–10, pre-lesion tests were conducted in the same manner as the differential training on days 5–7. On day 11, trained chicks were randomly allocated to two groups, which received either sham or lesion surgery. The chicks were allowed to recover from the operation for 1–2 days, and underwent post-lesion tests on days 13–15, one in the single phase and another in the triplet phase. Response peck latency was measured in single cue trials with the S+ color, and in binary choice trials with the S+ and S- options. Trials in which the chicks successfully pecked at the rewarding S+ option were analyzed.

Surgical procedure for electrolytic lesioning

Chicks were anesthetized via an intramuscular injection of 0.4 ml of a ketamine-xylazine cocktail, which was a 1:1 mixture of 10 mg/ml ketamine (Daiichi Sankyo Co., Ltd., Tokyo, Japan) and 2 mg/ml xylazine (Sigma-Aldrich Co., LLC, St. Louis, MO, USA). Supplementary injections (0.1 ml) were given to maintain stable anaesthesia. The chicks were fixed to a stereotaxic apparatus (SR-5N, Narishige, Tokyo, Japan) at the head angle set at ca. 45°. Body temperature was maintained using a heating pad. The skin over the skull surface was incised, areas of the skull removed, and the dura mater was cut to expose the brain. I constructed steel electrodes from electrolytically sharpened insect pins (type #00, max 300µm thick; Shiga Konchu Co., Tokyo, Japan). The pins were coated with enamel paint (Tamiya Inc., Shizuoka, Japan), leaving the ca. 0.6 mm-long tip unpainted.

Lesions were conducted bilaterally in all experiments. For lesioning the Arco (Experiment 1 and 3), the electrode was vertically inserted toward the coordinates: 2.0–2.3 mm anterior from bregma, 5.5 mm lateral from the midline, and 4.5 mm deep from the brain surface. For lesioning the lateral Arco, the electrode was vertically inserted toward the coordinates: 2.0–2.3 mm anterior from bregma, 6.5–6.6 mm lateral from the midline, and 3.7–3.9 mm deep from the brain surface. For lesioning the Nido, the coordinates were: 2.0–2.2 mm anterior from bregma, 5.5 mm lateral from the midline, and 2.0–2.5 mm deep from the brain surface. For lesioning the TnA, the coordinates were: 0.9–1.0 mm anterior from bregma, 4.1 mm lateral from the midline, and 6.0–6.2 mm deep from the brain surface. For the sham control group, I anaesthetized the subjects, placed them into the stereotaxic apparatus, incised the brain surface, and inserted an electrode, but did not apply current.

Chicks received one lesion per hemisphere. I applied pulses with constant current and

alternating polarity (amplitude: ± 1.5 mA, pulse duration: 50 ms, repetition rate: 10 Hz, 1 min in duration) to the inserted pin electrode using an electric stimulator (SEN-3301 and isolating unit SS-403J, Nihon Kohden, Tokyo, Japan). A silver wire was placed on the caudal skull as a reference electrode. After applying current, the electrode was left in place for 5 min before withdrawing. I covered the incised skull with the skin flap using superglue (Aron Alpha®, Toa Gosei Co., Ltd., Tokyo, Japan). After the surgery, chicks recovered individually overnight, and were then housed with their former cagemates.

Histological examination of the lesion site

On the day after the final test, the chicks were deeply anesthetized via an intramuscular injection of approximately 0.8 ml of ketamine-xylazine cocktail, and transcardially perfused with a fixative (4% paraformaldehyde in 0.1 M PB, pH 7.4). The dissected brain samples were post-fixed in the same fixative for one day at 4 °C, and then cryoprotected in 20% sucrose-PBS for one day at 4 °C. The brain tissue was frozen at -80°C for storage, and cut into 50 μm frontal sections using a sliding microtome with a freezing unit (Yamato Kohki Industrial Co., Ltd., Saitama, Japan). In some cases, in order to prevent brain regions (such as telencephalon and diencephalon) to fragment, the samples were embedded in yolk and post-fixed in the same fixative for >2 days at 4 °C. The tissue was then cut into 100 μm frontal sections using a vibrating microslicer (DTK-1000, Dosaka EM, Kyoto, Japan). Sections were mounted, air-dried, stained with cresyl violet, dehydrated, cleared using xylene, and cover-slipped.

The coordinates were based on the stereotaxic atlas of the chick brain by Kuenzel and Masson (1988) (Kuenzel & Masson, 1988), and terminology adhered to the nomenclature

reform (Reiner *et al.*, 2004).

Data analysis

Measurements of behaviors

In Experiments 1 and 2, I assessed the following behavioral indices. (1) Running distance was given by the sum of the distance that the subject ran in each trial in the single #1, paired, and single #2 phases. (2) The synchrony index was given as the ratio of time in which both chicks stayed in the same side of the maze, divided by the total trial time. When the two chicks were perfectly synchronized, the synchrony index was 1.0 (in-phase synchrony). When two chicks moved independently, the index was at the chance level, i.e., 0.5 (asynchrony). (3) The patch use ratio was given as the proportion of time in which the subject stayed close (<10 cm) to the VI10 feeder, divided by the total stay time at both feeders. The running distance and the patch use ratio in the two single phase sessions were averaged giving rise to $\overline{\text{single}} = \frac{\text{single \#1} + \text{single \#2}}{2}$. In Experiment 3, I assessed the response peck latency of rewarding trials in the single and triplet phases.

Statistical analyses of behavior data

I focused on the post-lesion running distance, synchrony index, patch use ratio (Experiments 1 and 2), and response peck latency (Experiment 3) as response variables. Group: Arco, lateral Arco, Nido lesions and sham control in Experiment 1; the TnA lesion and sham control in Experiment 2; and the Arco lesion and sham in Experiment 3. Phase: single and paired in Experiments 1 and 2; single and triplets in Experiment 3.

For Experiments 1 and 2, I made inter-group comparisons of running distance and patch use ratio by a two-way analysis of variance (ANOVA) with repeated measures based on type III sums of squares. The synchrony index was compared using a one-way ANOVA (Experiment 1) or Student t-test (Experiment 2). For Experiment 3, I made inter-group comparisons of response peck latency using a two-way ANOVA with repeated measures based on type I sums of squares.

Post-hoc pairwise comparisons were made after a Holm correction. The significance level was set at $P = 0.05$. All statistical calculations were performed using R (version 3.1.3, Windows version) and the “ANOVAKUN 4.8.0” add-on (programmed by Dr. Ryuta Iseki, <http://riseki.php.xdomain.jp/index.php?ANOVA%E5%90%9B>).

To compare the number of pecks per grain (κ index), I adopted a non-parametric permuted Brunner-Munzel test according to the R script developed by Dr. Haruhiko Okumura (Mie University, Tsu-city, Mie, Japan; <http://oku.edu.mie-u.ac.jp/~okumura/stat/brunner-munzel.html>). Pairwise comparisons were made among groups. P value was shown after Bonferroni correction.

2.3 Results

Sixty-seven chicks underwent surgical operations. Of these, four chicks were discarded in Experiment 1, as three chicks died during or after the surgery, and one stopped foraging. One and two chicks were similarly discarded in Experiments 2 and 3, respectively. The present results are based on data obtained from the remaining 60 chicks. All of these chicks actively foraged in their homecage and the apparatus, maintaining normal postures and locomotor activities throughout the experiments, including post-lesion.

Histology

In **Figure II-2A**, ablated areas are superimposed for 5 groups of chicks examined in Experiments 1, 2, and 3. For the Arco, the tissue damage included the intermediate Arco, dorsal Arco, and part of the lateral Arco, but the TnA appeared undamaged (**Figure II-2Bb**). The Nido was also partially lesioned in some of the chicks. For the lateral Arco, the lateral Arco was fully ablated in all chicks, while the dorsal Arco and intermediate Arco were partially damaged in some chicks. For the Nido, damage was localized to the areas dorsal to those lesioned in the Arco chicks. For the TnA, the bilateral lesion was successful, sparing the Arco (**Figure II-2Bc**).

Behavioral effects of Arco lesion (Experiment 1)

Chicks actively ran back and forth between the two patches. Representative trajectories of chicks with sham (n=11), Arco (n=7), lateral Arco (n=6), and Nido (n=7) lesions are shown in **Figure II-3**. Examples from the first session on post-lesion day 1 are included. In the

single phase, chicks in all groups tended to stay longer at the yellow patch (bottom) compared with the red patch (top), suggesting biased patch use. Note that the food delivery timing (arrowheads) did not strictly entrain the chick behaviors. In the paired phase, both the sham chicks and chicks with Nido lesions exhibited facilitated running and a high degree of synchrony. However, the chicks with Arco lesions were slower in the single phase, and often desynchronized in the paired phase. The chicks with lateral Arco lesions were also desynchronized in the paired phase, but reduced running was not apparent in the single phase.

In **Figure II-4Aa,b**, running distance and patch use ratio are plotted along eight successive sessions, four pre-lesion and four post-lesion. In the pre-lesion sessions, the running distance increased in the paired phase compared with the single #1 and #2 phases. The patch use ratio was slightly higher in the paired vs. the single phases. In the post-lesion tests, chicks with Arco lesions (green) exhibited a reduced running distance in both phases. Chicks with lateral Arco lesions (orange) showed a selectively suppressed running distance in the paired phase. On the other hand, chicks with Nido lesions (blue) behaved similarly to the sham controls. I found no significant differences in patch use ratio among the groups of chicks in the post-lesion tests.

I further compared the running distance data in the post-lesion tests among the four groups of chicks in the two phases (**Figure II-4Ac**); data in the single #1 and #2 phases were merged to give an average ($\overline{\text{single}}$). A two-way ANOVA revealed significant main effects of group ($F_{3,27} = 9.450$, $P = 0.0002$), phase ($F_{1,27} = 264.604$, $P < 0.0001$), and the group $\times$ phase interaction ($F_{3,27} = 17.221$, $P < 0.0001$). Multiple comparisons failed to reveal significant differences in the $\overline{\text{single}}$ phase data, but a suggestive level of difference

between the Arco vs. sham groups ($t = 2.744, P = 0.0640$) should be noted. In the paired phase, I found significant differences between the Arco vs. sham ($t = 6.159, P < 0.0001$), Arco vs. Nido ($t = 5.347, P = 0.0001$), lateral Arco vs. sham ($t = 3.465, P = 0.0072$), and lateral Arco vs. Nido ($t = 2.945, P = 0.0197$) groups, but not between the sham vs. Nido ($t = 0.248, P = 0.8063$) or Arco vs. lateral Arco ($t = 2.192, P = 0.0744$) groups.

The synchrony index data are compared in **Figure II-4Ad**. A one-way ANOVA detected a significant effect of group ($F_{3,27} = 11.076, P = 0.0001$). Multiple comparisons by Holm's tests revealed significant differences between the Arco vs. sham ($t = 4.132, P = 0.0016$), Arco vs. Nido ($t = 4.218, P = 0.0015$), lateral Arco vs. sham ($t = 3.901, P = 0.0017$) and lateral Arco vs. Nido ($t = 4.019, P = 0.0017$) groups, but not between the sham vs. Nido ($t = 0.033, P > 0.9999$) or Arco vs. lateral Arco ($t = 0.531, P > 0.9999$) groups.

The patch use ratios are compared in **Figure II-4Ae**. A two-way ANOVA detected significant main effects of phase ($F_{1,27} = 4.759, P = 0.0380$), but not group ($F_{3,27} = 0.226, P = 0.8778$) or group $\times$ phase interaction ($F_{3,27} = 0.786, P = 0.5122$).

The κ index (number of pecks per grain) was compared in four groups of chicks in Experiment 1; 1.12 ± 0.04 (mean $\pm$ SEM, sham, $n = 7$; median = 1.10), 29.01 ± 20.05 (Arco, $n = 5$; median = 6.50), 2.70 ± 0.48 (lateral Arco, $n = 6$; median = 2.59), 1.07 ± 0.02 (Nido, $n = 7$; median = 1.08). I did not measure the index in some chicks in sham and Arco groups. It is also to be noted that the large SEM value in the Arco group was due to 2 chicks with exceptionally high κ index (107.50 and 25.11). Permuted Brunner-Munzel test revealed significant difference between Arco vs. sham ($P = 0.0152$), and marginally significant

difference between lateral Arco vs. sham ($P = 0.0490$), but not between Nido vs. sham ($P > 0.9999$).

Behavioral effects of TnA lesion (Experiment 2)

The effects of the TnA lesion ($n=6$) on running distance are shown in **Figure II-4B**. Food delivery was not biased in Experiment 2. In terms of running distance (**4Ba**), a two-way ANOVA revealed significant main effects of phase ($F_{1,15} = 966.044$, $P < 0.0001$), but not group ($F_{1,15} = 1.916$, $P = 0.1866$) or interaction ($F_{1,15} = 0.196$, $P = 0.6641$). Student t-tests did not reveal a significant difference in synchrony indices (**4Bb**) ($t_{15} = 1.525$, $P > 0.1481$). In terms of the patch use ratio (**4Bc**), I found no significant effects of group ($F_{1,15} = 1.923$, $P = 0.1857$), phase ($F_{1,15} = 1.717$, $P = 0.2098$), or interaction ($F_{1,15} = 0.0068$, $P = 0.9354$).

Effects of Arco lesion on peck latency (Experiment 3)

The peck latency did not differ between the group of chicks with the Arco lesion ($n=6$) and the sham controls ($n=6$; **Figure II-4C**). A two-way ANOVA detected a significant main effect of phase ($F_{1,10} = 38.377$, $P = 0.0001$), but not group ($F_{1,10} = 0.525$, $P = 0.4853$) or interaction ($F_{1,10} = 1.494$, $P = 0.2496$).

2.4 Discussion

Bilateral electrolytic lesions of the Arco and lateral Arco significantly suppressed social facilitation of the foraging effort (**Figure II-4Aa, c**), but had no effect on matching behavior or peck latency (**Figure II-4Ab, e; C**). The effect of the lateral Arco lesion was selective, as it was not accompanied by changes in the running distance in the single phase. On the other hand, TnA lesion had no effects (**Figure II-4B**), though it is noted that the task of Experiment 2 is slightly different from Experiment 1. In the following, I will discuss the neural substrates responsible for social facilitation.

The high κ index found in the Arco and lateral Arco groups warrants a careful consideration on the lesion effects on running distance. When consuming food, chicks usually peck 2-4 times per sec in average (Matsushima *et al.*, 2008). In Experiment 1 of the present study, chicks were given two grains of millet at a time. Therefore, in these chicks with a high κ value, the consumption time (involved in the patch stay time) could be inevitably longer when chick encountered food, thus reducing the time for running. The suppressed running time in the single condition in the Arco group of chicks could be, at least partially, due to a side effect of the pecking impairment (**Figure II-4Ac**). The lateral Arco chicks showed a suppressed social facilitation without effects on the running distance, even though the κ index was significantly lower. These results are in concert with the idea that Arco plays a functional role in oro-facial motor control (Wild *et al.*, 1985; for further discussions, see below). On the other hand, the observed suppression in the social facilitation by the lesion cannot be accounted for by the pecking impairment that accompanies.

Social facilitation

A straightforward interpretation of the present lesion study is that the Arco, particularly the lateral region, enables chicks to overcome the extra effort that must be paid in a social foraging context. Most probably, the lateral Arco corresponds to the amygdalopiriform area of the Arco (abbreviated as APir; Puelles *et al.*, 2007, Hanics *et al.*, 2016), which is characterized by its dense projections to the nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BSTl; also see Chapter III). Previous study revealed that Arco lesions resulted in handling-cost aversion in a binary choice task, in which the larger option was accompanied by more pecks (Aoki, Csillag *et al.*, 2006). In present study, I focused on the foraging effort involved in approaching a food patch (Ogura & Matsushima, 2011; Ogura *et al.*, 2015), and found that chicks paid extra effort, even when it was not accompanied by extra food gain.

However, it would be premature to argue that the lateral Arco is specifically involved in social facilitation. First, no distinctive cytoarchitectonic differences have been found among the presumed regions of the Arco, despite the results of the present tracing study. Second, electrolytic lesions cannot unequivocally distinguish the contribution of the lateral Arco. Most probably, the lesions placed in the dorsal and intermediate Arco also damaged the efferent fibers issued from the lateral Arco, as has been reported previously (Atoji *et al.* 2006, Hanics *et al.* 2016). Additionally, lesions in the lateral Arco appeared to also affect the medial Arco regions to some degree. It should also be noted that as I observed only a partial loss of social facilitation after a considerable part of the Arco was lesioned, the Arco could be one of a number of involved substrates, each of which partially contributes to social facilitation.

An alternative interpretation of the present lesion study is that the Arco contributes to the visual perception of conspecifics, rather than foraging effort. This idea is compatible with the recent finding that visual exposure to alive conspecific activated amygdaloid nuclei in visually naïve chicks (Mayer, Rosa-Salva *et al.* 2017). If it is the case, we may assume that the observed suppression in facilitation is due to the incapability of the lesioned chick to discriminate companion chicks. However, this idea is not compatible with the finding that Arco lesions failed to suppress the shortened peck latency (**Figure II-4C**). Although the Arco might be involved in social perception, it is not critically required in all aspects of inter-individual interactions.

The Arco lesion might have directly suppressed locomotor activity, although I did not notice any deficiencies in postural or motor control in the lesioned chicks. The Arco is a heterogeneous structure comprising a somato-motor region and a limbic region (Wild *et al.*, 1985; Veenman *et al.*, 1995). The somato-motor region, including the intermediate and dorsal Arco, is thought to be analogous to the mammalian premotor/motor cortex (Shanahan *et al.*, 2013). This region is also involved in sensori-motor control (Zeier, 1971), specifically oro-facial control (Wild *et al.*, 1985). In pigeons, lesioning the intermediate Arco caused deficient feeding, and lesioned subjects could not hold grains with their beaks (Zeier, 1971). The increase in κ index found in the present study is in a good concert with these report.

More specifically, I may argue that the Arco lesion reduced the maximum limit of the running speed, making social facilitation less distinct. The reduced running in the single phase (**Figure II-3**) might thus be linked to the diverse projections of the medial Arco regions to midbrain areas including the tegmentum and TeO (see Chapter III, **Figures III-2, -3**). Indeed, reduced action in the descending reticulo-spinal pathway (see review by Grillner,

2006) may have slowed the running speed of the chicks after the Arco lesion. However, the lateral Arco did not project to the LSt (category $\eta 1$) or the tectum/tegmentum ($\eta 3$) (see Chapter III, **Figures III-2**).

The Nido is not considered to be involved in social facilitation, despite its dense projections to the MSt and LSt (category $\eta 1$) (see Chapter III, **Figures III-2**). Previous lesion (Diekamp, Gagliardo, *et al.*, 2002) and single-unit recording studies (Diekamp, Kalt, *et al.*, 2002; Veit *et al.*, 2014) in birds suggest that the caudo-lateral part of the Nido (NCL) is critical for working memory, similar to the prefrontal cortex (PFC) in mammals. However, our behavioral paradigm in the present study does not require working memory. Furthermore, the lesioned Nido area in chicks may be dissimilar from the NCL region studied in pigeons. It may be necessary to further characterize more lateral and caudal Nido areas.

The TnA lesion was also not effective, although previous studies have suggested that the TnA has amygdaloid features in birds (Cheng *et al.*, 1999; Absil *et al.*, 2002). Starlings with TnA lesions showed reduced “social facilitation” of foraging behavior in terms of behavioral synchronization (Cheng *et al.*, 1999), although these findings were not replicated in the present study. We should also notice that it remains controversial about the location and identification of nucleus taeniae in the avian brain (Puelles *et al.*, 2007, Hanics *et al.*, 2016).

Foraging effort investment

Ogura *et al.* (2015) reported a dissociation of the neural substrates involved in foraging effort and social facilitation of foraging. Specifically, lesioning the MSt suppressed foraging effort in the single phase, but did not lead to impaired social facilitation. The present tracing

results show that the lateral Arco projects to the NAc and MSt, whereas the intermediate and dorsal Arco project mainly to the MSt (see Chapter III, **Figures III-2**). A recent neuroanatomical study also revealed amygdalo-fugal terminals in the MSt/NAc with the morphological features of excitatory synapses (Hanics *et al.*, 2012). Taken together, these data indicate that Arco-MSt/NAc pathways are involved in determining foraging effort.

Matching behavior

The patch use ratio matched the biased food delivery rate between the two patches (**Figure II- 3 and 4Ab**) (also see Chapter I), and was unchanged by the lesions. Thus, the mechanisms that evaluate food patches, and proportionately allocate stay time, appear to be distinct from those involved in social facilitation. A similar dissociation was found by Aoki *et al.* (Aoki, Csillag *et al.*, 2006), who found that handling-cost aversion occurred without impairments to amount-based choices. To our knowledge, however, the neural mechanisms of matching have not been addressed in birds. In mammals, neurons in the parietal cortex represent the relative value of behaviors in a dynamic foraging environment (Sugrue *et al.*, 2004). However, the avian counterpart to the mammalian parietal cortex has not been established (see below for further comparative arguments). In primates, activity of phasically active neurons (PANs) in the primate striatum co-vary with the action-value, suggesting that PANs participate in the encoding of action values (Lau & Glimcher, 2008). The striatal pathways described above may also be involved in matching in chicks.

Operant peck latency

The Arco lesion did not change the peck latency (**Figure II-4C**). The response latency

is often supposed to represent the subjective value of a predicted food reward (Lauwereyns & Wisniewski, 2006). In chicks, however, experimental manipulation of handling-cost did not affect the peck latency, indicating that the latency does not represent the accompanying cost (Aoki, Csillag *et al.*, 2006). Most probably, the peck latency is socially facilitated by distinct mechanisms that are responsible for the foraging effort. In rats, dopamine depletion in the caudate nucleus has been associated with an increased response latency (Amalric & Koob, 1987). In future research, similar depletion effects in the striatum (LSt, MSt, and NAc) could be examined with respect to peck latency in chicks.

2.5 Figures

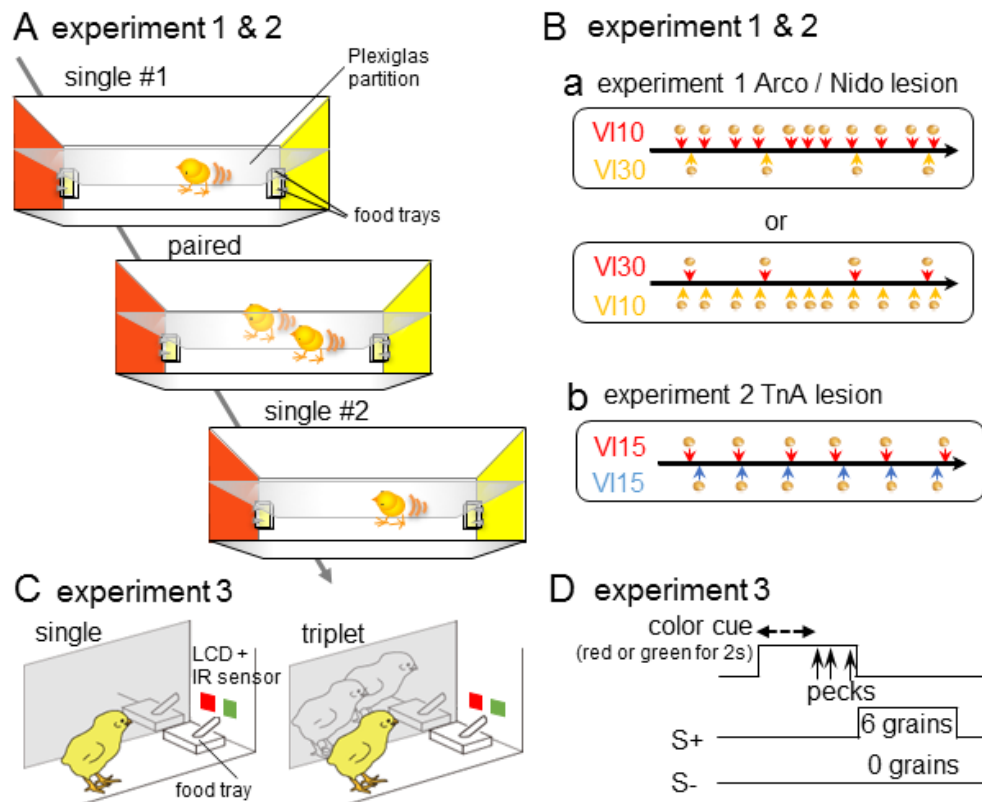


Figure II-1. Experimental apparatus and behavioral procedures for foraging behavior (A, B) and operant responses (C, D). (A) Apparatus and procedure of Experiments 1 and 2. An I-shaped maze was separated by a transparent Plexiglas partition to two lanes, each equipped with two food trays on both sides. One test block comprised three consecutive phases: single #1, paired, and single #2. (B) Examples of the food delivery schedules with arrowheads indicating the timing of delivery. In Experiment 1, the delivery was asymmetric as one patch followed the VI10 schedule (variable interval with the mean = 10 sec), and the other the VI30 (with the mean = 30 sec). Colors were randomly assigned among individuals. In Experiment 2, the delivery was symmetric and both patches followed the VI15 schedule.

(C) An operant box used in Experiment 3. Visual stimuli (red or green rectangles) were displayed on a LCD monitor, and an infra-red (IR) touch sensor detected chick pecking behavior. Chicks were tested either individually or in groups of three. In the triplet phase, the subject was separated from the two companions by transparent Plexiglas. (D) Trial procedure in Experiment 3. Color cues were presented for 2 sec. When the subject pecked at the rewarding cue, 6 grains of millet were delivered to the central food tray. No food followed when the subject pecked the non-rewarding cue.

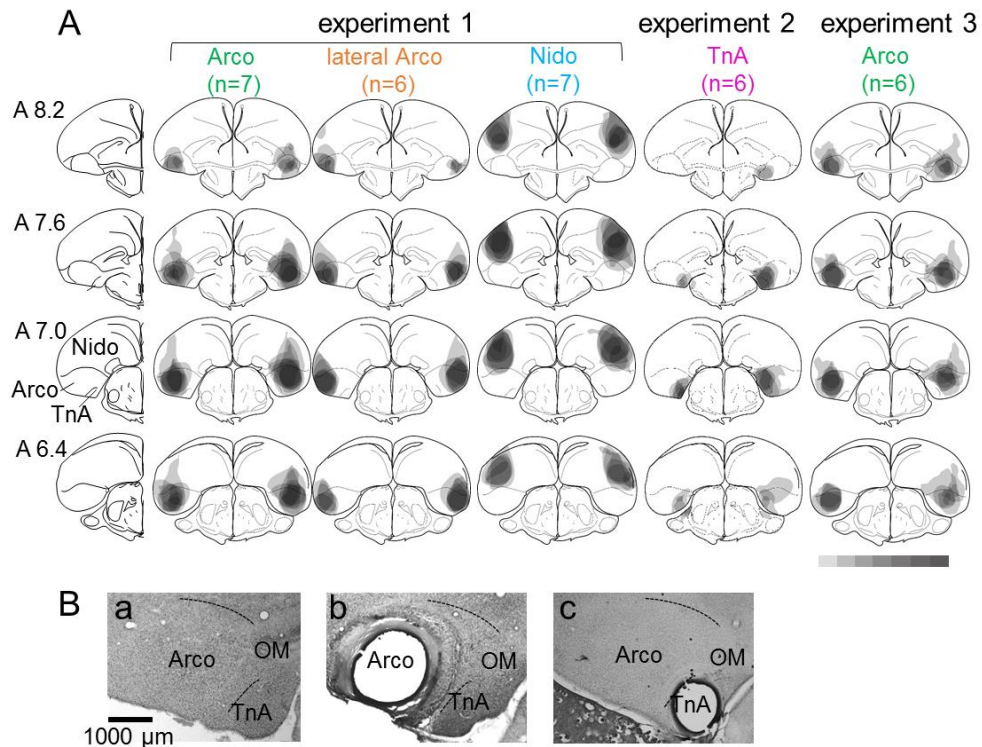


Figure II-2. Histological reconstruction of lesions. (A) Areas with damaged tissue are superimposed onto frontal sections. The levels of the sections (A 6.4 to A 8.2) follow the chick atlas by Kuenzel & Masson (1988). n denotes the number of chicks in each group. (B) Examples of Nissl-stained sections from the sham (a), Arco lesion (b), and TnA lesion (c) groups. See Abbreviations.

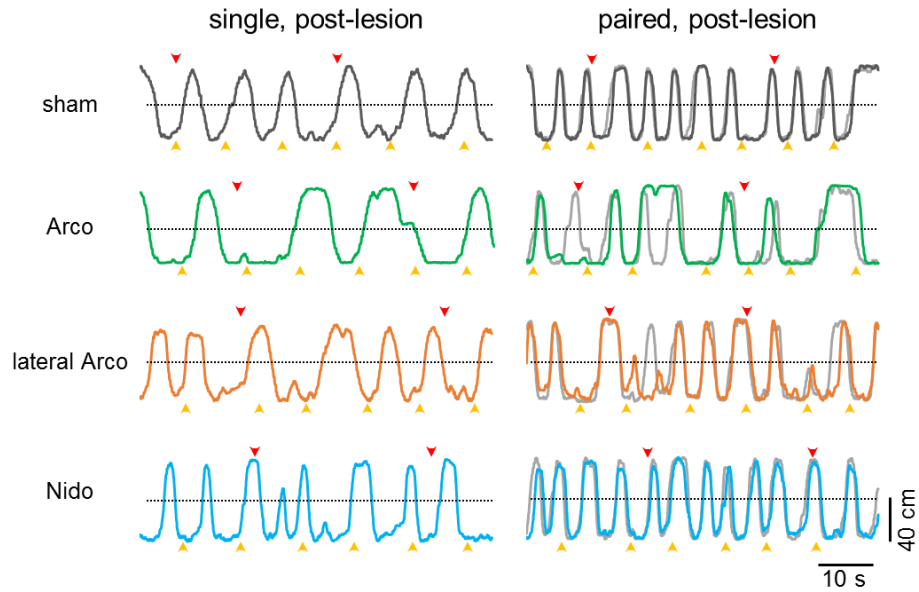


Figure II-3. Representative running trajectories of chicks with sham (dark grey), Arco (green), lateral Arco (orange), and Nido (blue) lesions. Trajectories from single subjects in the single (left) and paired (right) phases are shown. The trajectory of the naïve companion is superimposed in light grey. The y-axis indicates the position along the I-shaped maze (red: top, yellow: bottom), and the x-axis represents time. Horizontal dashed lines indicate the midpoint of the maze. Arrowheads denote the timing of food delivery. The delivery was biased, as the yellow patch followed the VI10 schedule (bottom) while the red patch followed the VI30 schedule (top).

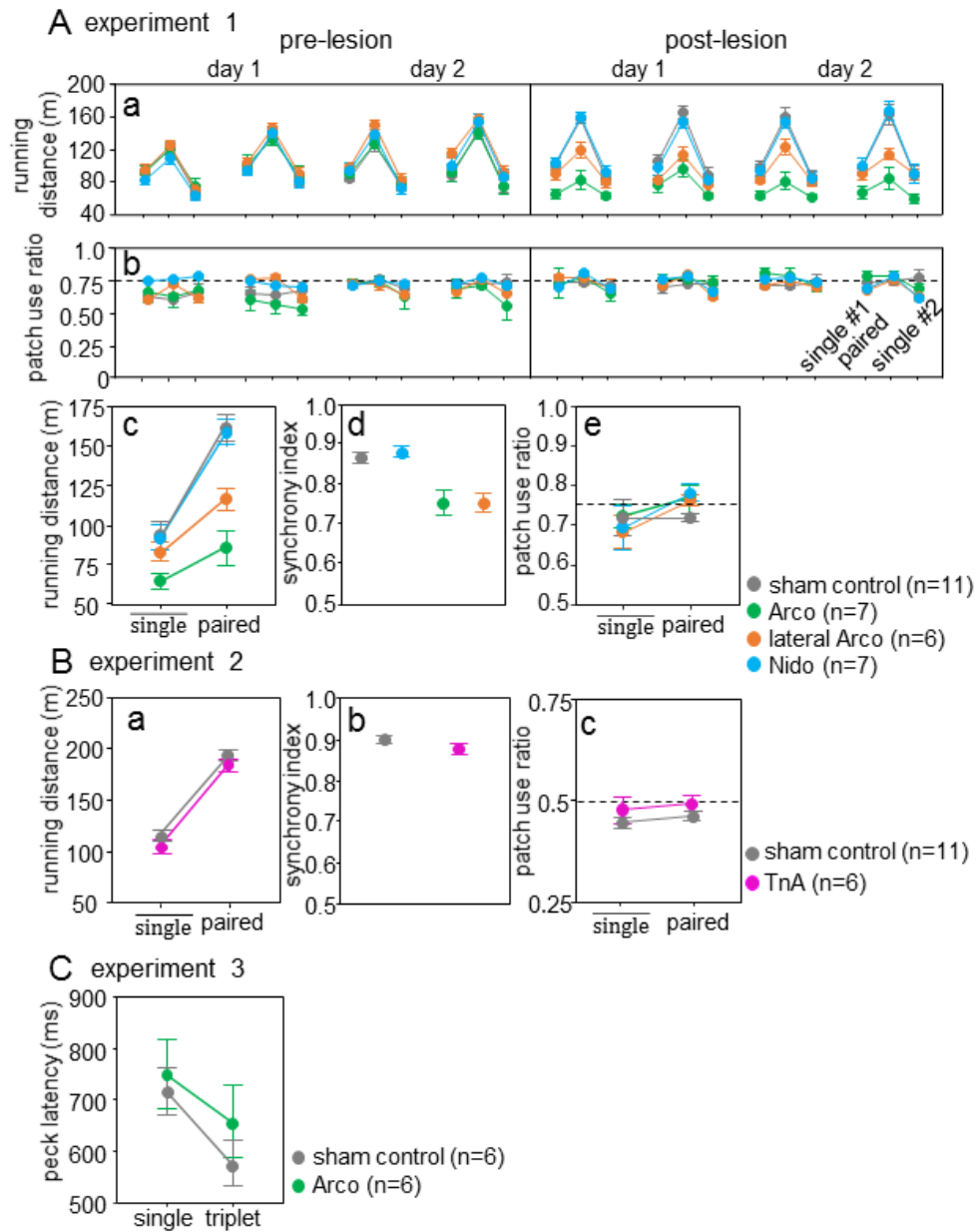


Figure II-4. Behavioral effects of lesions. (A) Effect of the Arco (green), lateral Arco (orange), and Nido (blue) lesions compared with the sham control (grey) in Experiment 1.

(a) Averaged running distance plotted against the sessions, which were each composed of three consecutive phases: single #1, paired, and single #2. Mean $\pm$ SEM are shown in this and the following graphs. (b) Patch use ratio at the VI10 feeder plotted against the sessions. Dashed horizontal lines indicate the level of 0.75. (c-e) Post-lesion data (4 sessions) compared among the four groups of chicks. (c) Average running distance in $\overline{\text{single}}$ (mean of single #1 and single #2) and paired trials. (d) Averaged synchrony index during the paired phase. (e) Patch use ratio in $\overline{\text{single}}$ (mean of single #1 and single #2) and paired trials. (B) Effect of the TnA lesion (pink) compared with the sham control (grey) in Experiment 2. (a) Running distance, (b) synchrony index, and (c) patch use ratio. Dashed horizontal lines in (c) indicate the level of 0.5. (C) I compared the effect of the Arco lesion (green) with the sham control (grey) in Experiment 3. Operant peck latency (ms) in rewarding trials. See text for statistical tests.

CHAPTER III

***Neuroanatomical tract tracing study: distinct projection
pattern of the lateral and medial arcopallium***

3.1 Introduction

Arcopallium (Arco, previously archistriatum) is a major descending area in the basolateral caudal telencephalon of avian brain. Although the organization of Arco is yet unclear, a widely accepted idea is that Arco is a heterogeneous structure comprising a limbic region and a somato-motor region (Wild *et al.*, 1985; Veenman *et al.*, 1995).

Report by Zeier and Karten (1971) was the first to make comprehensive neuroanatomical examination of the Arco in a non-songbird. They investigated the afferents and efferents of the pigeon Arco by combining lesions and silver impregnation techniques for demonstrating degenerating nerve fibers and terminals. Based on the results, they suggested that the Arco could be divided into four major regions, of which the posterior and medial Arco may be comparable to the mammalian amygdala and the anterior and intermediate Arco are associated with the sensori-motor system (Zeier & Karten 1971). Subsequently, the efferent connections of the chick Arco was investigated by using anterograde tracer phaseolus lectin (Davies *et al.* 1997). According to their study, Arco can be divided into limbic Arco and non-limbic Arco. The limbic Arco includes the posterior, ventral intermediate and anterior Arco. On the other hand, the non-limbic Arco which largely send specific efferents to sensory, somatosensory, and motor areas, comprises the dorsal intermediate and medial Arco (Davies *et al.* 1997). Furthermore, a recent computational neuroscience study based on brain connectivity revealed that the intermediate and dorsal Arco comprised somato-motor Arco, which is analogous to the mammalian premotor/motor cortex (Shanahan *et al.*, 2013). Compared with this, posterior and medial Arco are more limbic.

However, of these previous studies, the lateral Arco has rarely been concerned. In Chapter II, I found that Arco lesion, particularly the lateral Arco lesion selectively suppressed social facilitation. Pathways from the lateral Arco could enable chicks to overcome the extra effort that must be paid in a social foraging context. I therefore examined efferent projections of Arco sub-regions by focal infusions of anterograde tracer (biotinylated dextran amine, BDA), and attempted to relate the efferent projections with our lesion data in Chapter II.

3.2 Materials and methods

Subjects and Ethical Note

I used male domestic chicks (*Gallus gallus domesticus*, White Leghorn strain). On post-hatch day 1 (presumed hatching day), I purchased new hatchlings from a local supplier (Iwamura poultry Ltd. Hokkaido establishment, Yubari, Hokkaido, Japan). Chicks were paired and communally housed in transparent plastic cages (15 cm × 28 cm × 12 cm) in a room illuminated according to a 12:12-h light:dark cycle with the light period starting at 08:00 a.m. The temperature of the room was kept constant at ca. 30 °C.

The chicks received two types of food, i.e., grains of millet and chick mash food. The total amount of food per day was adjusted so that (1) the body weight of the chicks gradually increased and (2) the chicks actively consumed food during experiments. Chicks were given mash food from post-hatch day 1. Specifically, they received 2 g (post-hatch days 1–3) and 2.5 g (day 4 and afterwards) of mash food. Grains of millet were added from post-hatch day 2. The amounts of grains were 2 g (days 2–3) and 2.5 g (day 4 and afterwards). Until post-hatch day 2, all chicks were communally fed. From post-hatch day 3, chicks were individually fed in cages that were visually separated by black boards. Water was freely available.

The experiments were conducted under the guidelines and approval of the Committee on Animal Experiments of Hokkaido University. The guidelines are based on the national regulations for animal welfare in Japan (Law for Humane Treatment and Management of Animals; after a partial amendment No. 68, 2005). After the experiments, the brains of the chicks were dissected under deep anaesthesia. In cases in which surgical operations were not

conducted, the chicks were euthanized with carbon dioxide.

Injection of BDA

To examine the efferent terminals projecting from different sites within the Arco, I used biotinylated dextran amine (BDA, 10% in distilled water, 10 kDa; D22910, Molecular Probes®, Thermo Fisher Scientific Inc., Waltham, MA, USA) as an anterograde tracer. I used 15 chicks: 13 for different sites within the Arco, and 2 for the Nido. On approximately post-hatch day 7, I injected BDA using a Nanoject II (Drummond Scientific Co., Broomall, PA, USA) under ketamine-xylazine anaesthesia, as described in the methods for the lesion experiment. The tracer was deposited via a slow pressure injection lasting for 10–15 min (13.8 nl per injection $\times$ 11 injections, 150 nl per site). After the injection, the glass capillary was left in place for an additional 10 min to minimize leakage of the dye along the injection track.

Perfusion and sectioning

Seven days after the BDA injection, chicks were deeply anaesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), as in the post-lesion histological examination. Dissected brains were post-fixed, cryoprotected, and sectioned into 50- μ m sections on a frontal plane using a freezing microtome. Sections were stored in 4°C phosphate-buffered saline (PBS) for histochemical processing.

Histochemical processing of BDA-labelled terminal fibers

Sections were pretreated in 3% H₂O₂ in PBS (pH 7.4) for 30 min, rinsed in PBS (4×), incubated in avidin-biotinylated horseradish peroxidase complex (ABC, Vectastain® Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 1 hr at room temperature, and then rinsed in PBS (4×) and in distilled water (1×). BDA labelling was developed using nickel-enhanced diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride, DAB, DAB Peroxidase Substrate kit, SK-4100, Vector Laboratories). The sections were incubated in distilled water containing Tris buffer, DAB, H₂O₂, and nickel-ammoniumsulphate (1:2:1:1) for 5–10 min. The reaction was visually controlled and terminated by rinsing the sections in PBS (3×). After the staining was developed, the sections were mounted onto amino silane - coated glass slides (Matsunami glass Ind., Ltd., Japan), air-dried, dehydrated, cleared, and coverslipped.

Quantitative analysis of BDA projection patterns

Labelled fibers and terminal arborizations were localized according to the brain atlas (Kuenzel & Masson, 1988; Puelles *et al.*, 2007). Further abbreviations of neural nuclei and nuclei boundaries followed the nomenclature reform (Reiner *et al.*, 2004) and also the anatomical data reported by Montagnese *et al.* (2003) and Hanics *et al.* (2016). Based on visual inspections of the intensity of labels, blind to the injection site, I determined five grades of fiber density: abundant, moderate, low, sparse (only 2–3 fibers in the entire region), and absent. I used hierarchical clustering analysis (Everitt *et al.*, 2011) with the Euclidean metric and the average agglomeration method to create clustering of the efferent regions. I generated a heatmap with dendrogram classification using the “pheatmap” package (version 1.0.8) of

R statistics.

3.3 Results

Sixteen chicks received BDA micro-injections, of which 15 showed successful staining. **Figure III-1A** shows representative injection sites in three chicks, and **Figure III-1B–D** show anterogradely labelled fibers with dense varicosities. Retrogradely labelled cell bodies (**Figure III-1Cg**) were found in some cases (see below). Intensive labels appeared almost exclusively in the hemisphere ipsilateral to the injection site. In a few cases, however, sparse labels occurred in the contralateral midbrain. Furthermore, I found projections to the contralateral telencephalon (including the contralateral Arco) in one chick. In the following analyses, I disregarded these contralateral projections. After the BDA injection into the lateral Arco, I found dense terminal labels in the hippocampus (Hp, **Figure III-1Ba**), septum (Sept, **Figure III-1Bb**), and nucleus accumbens (NAc, **Figure III-1Bc**), as well as other regions. Injection into the dorsal Arco (**Figure III-1C**) yielded terminal labels in a wide range of areas such as the intermediate medial mesopallium (IMM, **Figure III-1Ca**), caudal extended amygdala (CEA, **Figure III-1Cb**), and ventral tegmental area (VTA, **Figure III-1Cc**), which was also labelled after the lateral Arco injection. On the other hand, I found labelling in the following areas after injection into the dorsal and the intermediate Arco: terminals in the medial spiriformis nucleus (SPM, **Figure III-1Cd**), the intercollicular nucleus (ICo, **Figure III-1Ce**), and the optic tectum (TeO, **Figure III-1Cf**). In addition, injection into the dorsal Arco yielded retrogradely labelled cell bodies in the Nido (**Figure III-1Cg**), whereas no such labels appeared after the lateral Arco injection. When BDA was injected into the Nido, anterograde labels were found in limited brain regions. These included the Arco, lateral striatum (LSt, **Figure III-1D**), MSt, rostral and caudal part of extended amygdala (rEA and

cEA).

The results of hierarchical cluster analysis of the efferent projection patterns are shown in **Figure III-2**. Chicks were categorized into three main clusters (arbitrarily labelled as α , β , and γ), each of which proved to correspond to the injection site. Chicks in cluster α had received a Nido injection ($n = 2$), and their projection patterns were distinct from those in the other groups. Chicks in cluster β corresponded to the lateral Arco ($n = 5$), and chicks in cluster γ included three sub-clusters; γ_1 for lesions in the PoA and ventral Arco, γ_2 for the dorsal Arco, and γ_3 for the intermediate Arco (see the injection sites below).

The regions of the efferent fibers and terminals were categorized into 4 clusters, δ , ϵ , ζ , and η (η_1 , η_2 , and η_3). Many telencephalic nuclei were allocated to clusters δ and ϵ . Some “limbic” structures such as the hippocampus (Hp) and septum (Sept) were included in cluster ϵ , which mainly received efferents from the lateral Arco. Chicks with BDA injection to the ventral Arco were grouped in cluster δ , but the terminals were sporadic and sparse if present. Brain areas categorized in the ζ cluster (rEA and cEA) received projections from both lateral and other Arco regions. Nuclei in the thalamus, hyper thalamus, tegmental areas, and optic tectum (TeO) were categorized in cluster η . The η_1 cluster included the LSt and MSt, the former of which selectively received efferents from the dorsal and the ventral Arco (or collectively, the medial Arco). It should be noted that the Nido strongly projected to the LSt (**Figure III-1D**). The LSt labels after the medial Arco injection could therefore be due to a diffusion of BDA from the injection site. The η_2 cluster included the midbrain central gray (GCt), thalamic anterior dorsomedial nucleus (DMA), thalamic posterior dorsomedial nucleus (DMP), thalamic anterior dorsolateral nucleus (DLA), thalamic posterior dorsolateral nucleus (DLP), and thalamic posterior dorsointermediate nucleus (DIP). The η_2

cluster received projections from both the lateral and more medial Arco regions. Midbrain areas such as the intercollicular nucleus (ICo) and optic tectum (TeO) were included in cluster η_3 , which selectively received projections from the medial Arco. In the Nido, retrogradely labelled cell bodies appeared only in those chicks that had received an injection in the medial regions of the Arco.

3.4 Discussion

Anterograde tracing revealed distinct patterns of efferents from Arco sub-regions (**Figure III-1 and 2**). Assuming that the Arco, especially the lateral Arco is selectively involved in social facilitation (see Chapter II), candidate Arco efferents involved in social facilitation can be suggested.

First of all, the extended amygdala (rEA and cEA) may contribute to this behavior, as they both receive dense projections from the Arco (**Figure III-2 and 3**). Currently, these regions are both recognized as part of the amygdaloid complex in the avian brain (Reiner *et al.*, 2004). Further research involving localized lesions to these amygdaloid nuclei may help to clarify this issue.

A projection from the lateral Arco to the NAc might be another candidate efferent (**Figure III-2 and 3**). In rats, pharmacological manipulation of dopaminergic activity in the NAc biases behavior away from costly actions without disrupting discrimination between rewards of different magnitudes (Salamone *et al.*, 1994). In chicks, electrolytic lesioning of the MSt/NAc suppressed foraging efforts, but dopamine depletion in these regions had no effect (Ogura *et al.*, 2015). A projection to the Sept may also be a candidate. In primates, a class of Sept neurons code reward uncertainty; these may process risk-cost and related emotional responses (Monosov & Hikosaka, 2013). Distinct projections from the lateral Arco to the Sept (**Figures III-1Bb, 2 and 3**) might play a critical role in the social foraging.

The involvement of a descending projection to midbrain tegmental areas is also an important consideration. As lesioning the tegmentum around the SN suppresses social facilitation (Ogura *et al.*, 2015), damage to the major descending fiber bundle (OM; Zeier &

Karten, 1971; Davies *et al.*, 1997) may be a critical factor. However, the lateral Arco only sparsely projects to the tegmental nuclei. The specific involvement of the lateral Arco-tegmental pathway is thus questionable.

Future research including localized pharmacological and/or molecular manipulations of the efferent (lateral) Arco pathways should be done to specify the efferents responsible for social facilitation.

3.5 Abbreviation

Abbreviation	English name
AMH	anterior medial hypothalamic nucleus
Apir	amygdalopiriform area
Arco	arcopallium
DIP	thalamic posterior dorsointermediate nucleus
DLA	thalamic anterior dorsolateral nucleus
DLP	thalamic posterior dorsolateral nucleus
DMA	thalamic anterior dorsomedial nucleus
DMP	thalamic posterior dorsomedial nucleus
EA	extended amygdala
GCt	midbrain central gray
GP	globus pallidus
HbL	lateral habenula
HbM	medial habenula
Hp	hippocampus
ICo	intercollicular nucleus
IH	inferioris hypothalamic nucleus
IMM	intermediate medial mesopallium
LSt	lateral striatum
Lhy	lateral hypothalamus
LoC	locus coeruleus

MLd	dorsal lateral mesencephalic nucleus
MSt	medial striatum
NAc	nucleus accumbens
NIII	oculomotor (third) nerve
Nido	nidopallium
OM	occipito-mesencephalic tract
OTu	olfactory tubercle
PHN	hypothalamic periventricular nucleus
PMI	thalamic nucleus paramedianus internus
POM	medial preoptic nucleus
PVN	paraventricular nucleus
Pap	papillioformis nucleus
PoA	posterior pallial amygdala
Ru	nucleus ruber
SC	nucleus subceruleus
SCE	stratum cellulare externum
SN	substantia nigra
SPL	lateral spiriformis nucleus
SPM	medial spiriformis nucleus
Sept	septum
TeO	optic tectum
TnA	nucleus taeniae of the amygdala

VL	lateral ventricle
VMN	hypothalamic ventromedial nucleus
VP	ventral pallidum
VT	third ventricle
VTA	ventral tegmental area
cBSTl	caudal bed nucleus of the stria terminalis
rBSTl	rostral bed nucleus of the stria terminalis

3.6 Figures

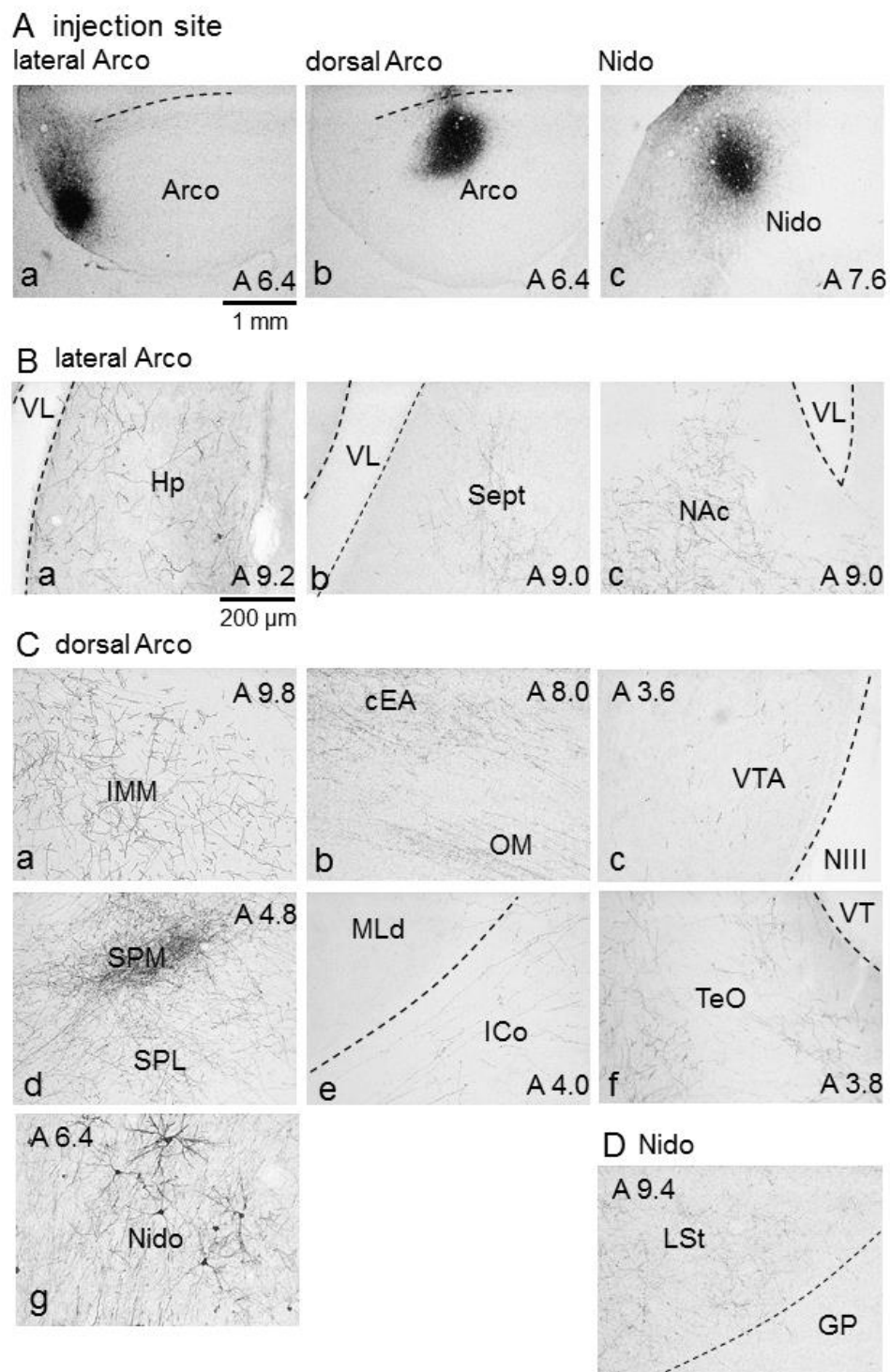


Figure III-1. Anterograde tract tracing using biotinylated dextran amine (BDA). (A) Examples of BDA injection sites in the lateral Arco (**a**), dorsal Arco (**b**), and Nido (**c**). Levels of the frontal sections follow the atlas by Kuenzel & Masson (1988). (B) Anterogradely labelled terminals in the hippocampus (Hp, **a**), septum (Sept, **b**), and nucleus accumbens (NAc, **c**) after injection in the lateral Arco. (C) Anterogradely labelled terminals in the intermediate medial mesopallium (IMM, **a**), caudal extended amygdala (cEA, **b**), ventral tegmental area (VTA, **c**), medial spiriformis nucleus (SPM, **d**), intercollicular nucleus (ICo, **e**), and optic tectum (TeO, **f**) after injection in the dorsal Arco. I also found retrogradely labelled cell bodies in the Nido (**g**). Dashed lines indicate the boundaries of the regions. (D) Anterogradely labelled terminals in the lateral striatum (LSt) after injection in the Nido. See Abbreviations.

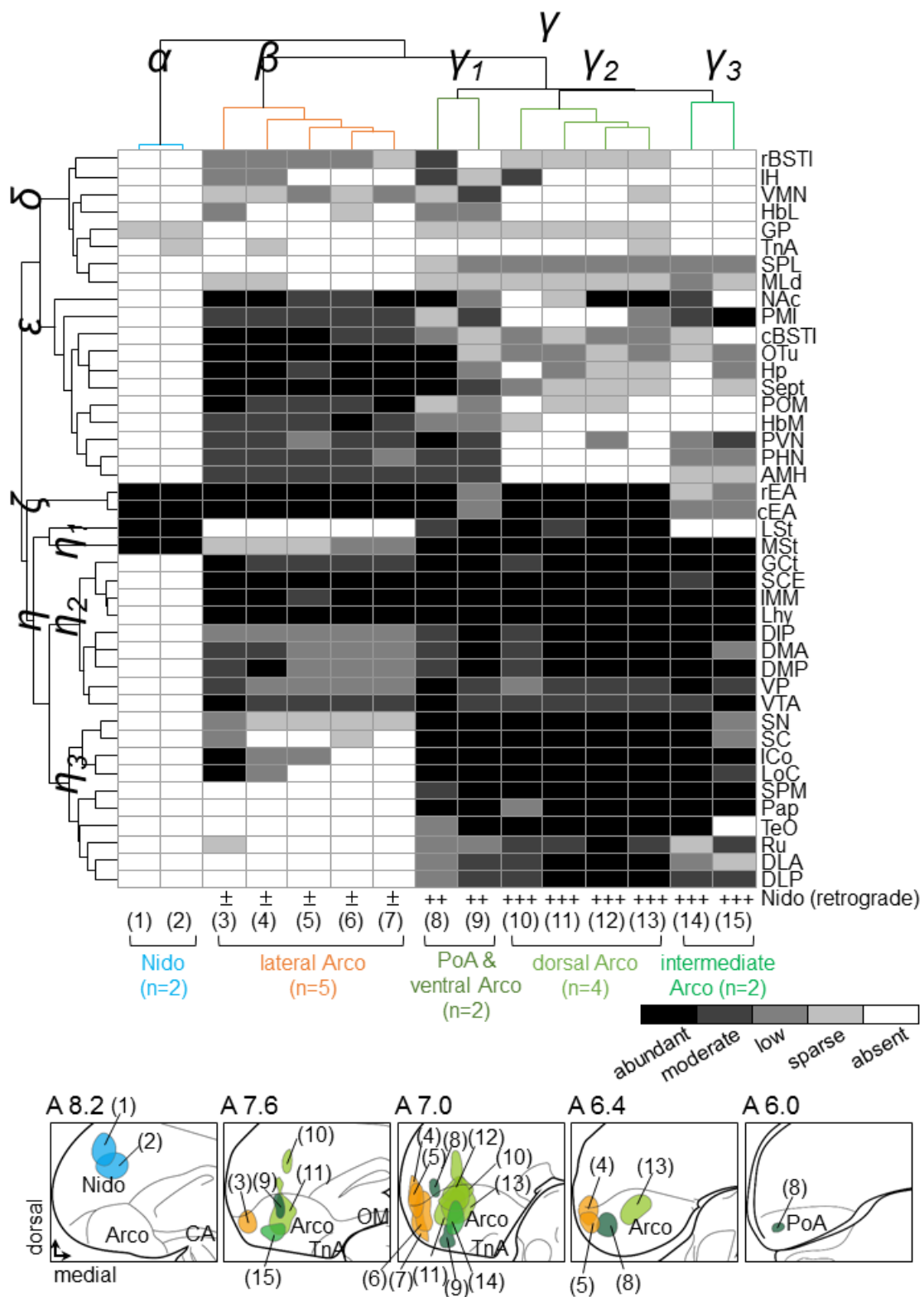


Figure III-2. Hierarchical clustering of efferent projections. Rows indicate the recipient brain regions, and columns represent the subjects. I semi-quantitatively determined five grades of terminal density: abundant, moderate, low, sparse, and absent. Subjects were grouped into three main clusters; α , β , and γ , and cluster γ was further divided into three sub-clusters: γ_1 , γ_2 , and γ_3 . The recipient brain regions were also grouped into four clusters δ , ϵ , ζ , and η , and cluster η was further divided into three sub-clusters, η_1 , η_2 , and η_3 ; where n denotes the number of chicks. At the bottom, retrogradely labelled cell density in the Nido is indicated as +++, ++, and $\pm$. See the text for details and see Abbreviations. The BDA injection sites are illustrated below. Different colors indicate different clusters of subject chicks and numbers in parenthesis (1~15) denote the injection site in individual chicks.

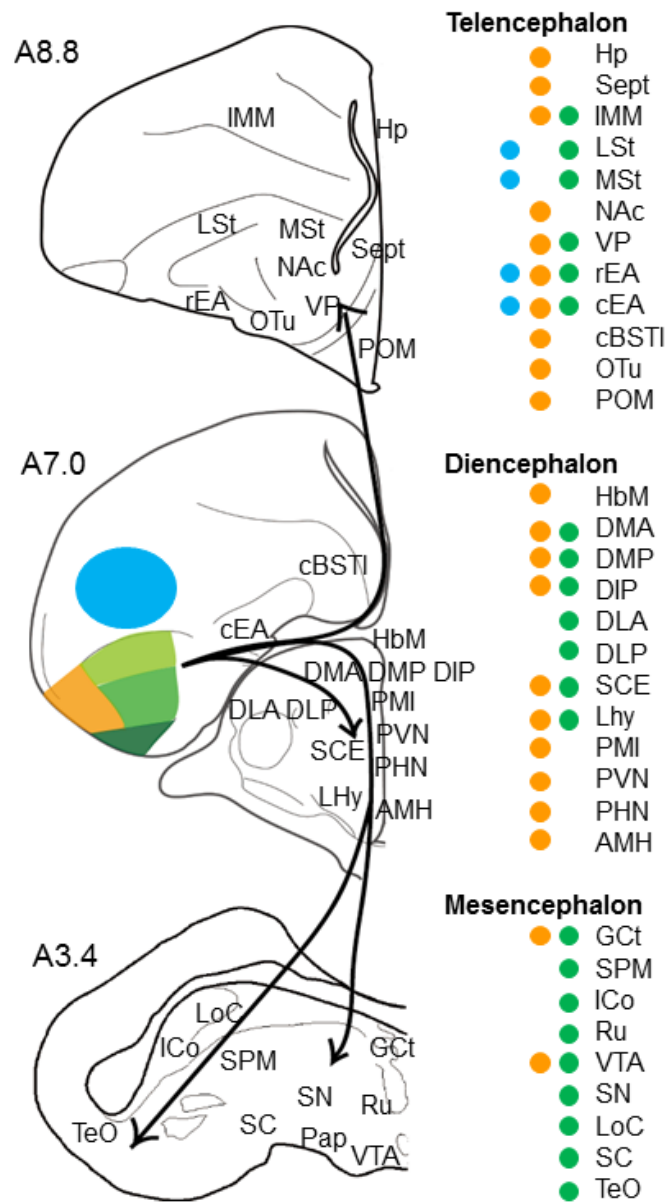


Figure III-3. Major efferent projections from sub-regions in the Arco. Recipient areas in the telencephalon (A8.8), diencephalon (A7.0), and midbrain (A3.4) are schematically shown. Projections from the lateral Arco (orange), medial regions of the Arco (green), and the Nido (blue) are labelled using the same colors in **Figure III-2**. Areas with abundant and moderate efferent terminals are shown on the right columns. Refer to the abbreviation table.

GENERAL DISCUSSION

*Is the avian arcopallium analogous to the prefrontal cortex
or amygdala in mammals?*

In summary, the Arco, particularly the lateral region, enables chicks to overcome the extra effort that must be paid in a social foraging context. Our findings in domestic chicks may give us a hint for understanding the possible correspondence between the avian and mammalian social neuro-economic systems.

Based on our lesion data (Chapter II), we may argue that the Arco plays multiple functions in a manner analogous to the mammalian prefrontal cortex, particularly the anterior cingulate cortex (ACC). Lesioning the mammalian ACC caused an effort-cost aversion, biasing behavior away from a costly action (Walton *et al.*, 2003, 2009; Schweimer *et al.*, 2005; Rudebeck *et al.*, 2006). As with the chicken Arco (Phillips & Youngren, 1986), a taming effect appeared after an ACC lesion in humans, specifically personality changes, such as lack of distress (Tow & Whitty, 1953; Cohen *et al.*, 2001). The avian Arco is involved in sensori-motor control (Zeier, 1971; Knudsen & Knudsen, 1996b), and this may hold true for the mammalian ACC. For instance, bilateral lesions of the rostral ACC is reported to cause oculomotor deficits, i.e., deficits in central gaze fixation (Paus *et al.*, 1991).

This issue should also be discussed in anatomical terms (Chapter III). As with the lateral Arco, the ACC/mPFC has connections with the hippocampal-parahippocampal region (Carmichael & Price, 1995; Chiba *et al.*, 2001; Kondo *et al.*, 2005; Mohedano-Moriano *et al.*, 2007; Passingham & Wise, 2012). In addition, the projections of lateral Arco to other limbic regions, such as the hypothalamus, dorsal thalamus, extended amygdala (rEA and cEA), medial striatum (MSt), and nucleus accumbens (NAc) should not be ignored. In the ACC, similarly strong connections appear with limbic regions such as the hypothalamus (Ongür *et al.*, 1998), amygdala (Morecraft *et al.*, 2007), and striatum (Haber *et al.*, 1995).

Such functional and anatomical considerations enable the alternative idea of comparing

the lateral Arco to the mammalian amygdala, particularly the basolateral amygdala (BLA). Inactivation of the BLA also causes effort-cost aversion (Floresco & Ghods-Sharifi, 2007). Lesions of the chicken Arco (Phillips & Youngren, 1986; Lowndes & Davies, 1995; Saint-Dizier *et al.*, 2009) and its electrical stimulation (Phillips & Youngren, 1971) have suggested that Arco is critical for fear- or anxiety-related behavior, similarly to the mammalian BLA in terms of emotion processing (Vazdarjanova *et al.*, 2001; Tye *et al.*, 2011).

Anatomically, as pointed out by Hanics *et al.* (Hanics *et al.*, 2016), the efferent projections from the lateral Arco to the NAc and bed nucleus of the stria terminalis (BSTl) suggest a correspondence to the mammalian BLA. Similarly, the BLA has projections to the hippocampal formation (Saunders *et al.*, 1988; Pitkänen *et al.*, 2000) and septum (Sept; Calderazzo *et al.*, 1996) in mammals. Based on common neurochemical characterization, Martinez-Garcia *et al.* (Martínez-García *et al.*, 2009) hypothesized that the Arco complex is homologous with the mammalian BLA.

Convergent evolution of “prefrontal” and “amygdala” functions between birds and mammals, together with their functional multiplicity and anatomical homologies, should be carefully examined in future.

ACKNOWLEDGEMENTS

Deepest gratitude for the help and support are extended to all the people who have contributed in making this study possible.

I firstly would like to express my sincerest gratitude to my supervisor Prof. Toshiya Matsushima to his enormous support. He has spent so much effort for teaching me and leading me to the scientific world of Neuroscience. I found my three and a half years here in Matsushima's laboratory all very fulfilling.

I am grateful to members of the dissertation refereeing committee, Prof. Makoto Mizunami (Hokkaido University), Dr. Kazuhiro Wada (Hokkaido University) and Prof. Michael Colombo (University of Otago, New Zealand) for giving me critical comments and suggestions.

I am also very grateful to Onur Güntürkün (Ruhr-Universität Bochum, Germany) for his critical comments and also for giving me an opportunity to visit his lab in Ruhr-Universität Bochum. I also would like to thank to András Csillag (Semmelweis University, Hungary) for his valuable comments on the anatomy.

I sincerely appreciate all the members of Matsushima laboratory and other members of laboratories of Behavioral Neurobiology. Especially I express my gratitude to Dr. Yukiko Ogura (now at Department of Psychiatry, Graduate School of Medicine, Hokkaido University), who designed the apparatus for behavioral task, conducted TnA lesion experiment and gave valuable comments and suggestions on my study. Thanks are also due to LeO Uno for conducting response peck latency experiment and offering me data.

I thank Mr. Naohumi Katou (Hokkaido University) for making apparatus. I am also appreciate Dr. Kazuhiro Wada for offering me tracers.

A final word of thanks is reserved for my parents, my elder brother and my boyfriend for their love and support, without their encouragement I would never have the courage to pursuit my scientific interest in Japan

This study was supported by a grant-in-aid from the Japan Society for the Promotion of Science (JSPS KAKENHI, Scientific Research (B) #25291071 and Challenging Exploratory Research #26650114) to Toshiya Matsushima.

I would like to acknowledge the financial support by the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT) through a scholarship for postgraduate study (from Oct. 2013 to Mar. 2017) which made my stay in Japan possible.

Thanks to Grant for Attending Meeting or Conference (BioSystem Science Course, Graduate School of Life Science, 2015) for partially offering travel fee to go to the annual conference of Society of Neuroscience (SfN, Chicago, USA, 2015). And thanks to Stipend for the Development of Future Researchers (Faculty of Science, 2015) and Grant for Globalization Projects (BioSystem Science Course, Graduate School of Life Science, 2016) which made my attending to the Forum of Neuroscience (FENS, Copenhagen, Denmark, 2016) and my visit to Ruhr-Universität Bochum (Germany, 2016) possible.

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LIST OF PUBLICATIONS

1. QiuHong Xin, Yukiko Ogura, Reo Uno, Toshiya Matsushima: “Selective contribution of the telencephalic arcopallium to the social facilitation of foraging efforts in the domestic chick”, *European Journal of Neuroscience*, 2017, **45**, 365-380
2. QiuHong Xin, Yukiko Ogura, Toshiya Matsushima: “Four eyes match better than two: patch use behavior of socially foraging chicks”, in preparation

LIST OF OTHER PUBLICATION

Dissertation related Presentations

International Congress (Oral presentation)

1. Q Xin & T. Matsushima

“Anatomy of Social Facilitation and Synchronization in Group Foraging Domestic Chicks”,
SWARM 2015: The First International Symposium on Swarm Behavior and Bio-Inspired Robotics, Kyoto (Japan), October, 2015

2. T. Q Xin & T. Matsushima

“Functional contribution of arcopallium in the social facilitation of foraging effort in domestic chicks: a lesion experiment and a tract tracing study”, *BioPsy-Colloquium (Ruhr-Universität Bochum)*, Bochum (Germany), July, 2016

International Congress (Poster presentation)

3. T. Ogura, Q Xin & T. Matsushima

“Involvement of substantia nigra but not the dopaminergic neurons in social facilitation of foraging efforts in domestic chicks”, *Joint meeting of the 11th International Neuroethology Conference (ICN) and 36th Annual Meeting of The Japanese Society for Comparative Physiology and Biochemistry (JSCPB)*, Sapporo (Japan), July, 2014

4. T. Ogura, Q Xin & T. Matsushima

“Foraging effort and its social facilitation in the domestic chick: double dissociation of medial striatum and substantia nigra”, *44th Annual Meeting of Society for Neuroscience*, Washington DC (USA), November, 2014

5. ○Q Xin & T. Matsushima

“Social facilitation of foraging effort in domestic chicks: functional contribution of the descending pathway from arcopallium to midbrain tegmentum”, *45th Annual Meeting of Society for Neuroscience*, Chicago (USA), October, 2015

6. ○Q Xin, Y. Ogura & T. Matsushima

“Functional contribution of arcopallium in the social facilitation of foraging effort in domestic chicks”, *10th FENS Forum of Neuroscience*, Copenhagen (Denmark), July, 2016

Domestic Congress (Oral presentation)

7. ○Q Xin & T. Matsushima

“Paired chicks persevere: social influences on foraging strategies and memory recall”, 日本動物学会第 85 回大会、仙台、2014 年 9 月

8. ○Q Xin & T. Matsushima

“Functional contribution of arcopallium in social facilitation of foraging effort in domestic chicks (*Gallus domesticus*)”, 日本動物学会北海道支部第 60 回大会、札幌、2015 年 8 月

9. M. Miura, Q Xin, Y. Ogura & ○T. Matsushima

“Socio-economics prototype in domestic chicks: interplays among biological motion, imprinting, and social facilitation”, 日本動物心理学会第 75 回大会、東京、2015 年 9 月

10. Q Xin, Y. Ogura, & ○T. Matsushima

“Social foraging in domestic chicks: contribution of descending pathway from arcopallium (isocortex)”, 日本動物学会第 86 回大会、新潟、2015 年 9 月

Domestic Congress (Poster presentation)

11. ○Q Xin & T. Matsushima

“Social facilitation of foraging effort in domestic chicks: functional contribution of the descending pathway from arcopallium to midbrain tegmentum”, 「脳と心のメカニズム」第 16 回 冬のワークショップ, 留寿都, 北海道, 2016 年 1 月

12. ○Q Xin, Y. Ogura & T. Matsushima

“Selective contribution of the telencephalic arcopallium to the social facilitation of foraging efforts in the domestic chick”, 日本動物心理学会第 76 回大会、札幌、2016 年 11 月

Dissertation unrelated Presentations

International Congress (Poster presentation)

13. ○Q Xin, Y Liu, R Hou, Z Cai, Z Zhang, J Lan, D Liu

“Reproductive advertisement: chemical signals in relation to reproductive status in the urine of female giant pandas (*Ailuropoda melanoleuca*)”, *Joint meeting of the 11th International Neuroethology Conference (ICN) and 36th Annual Meeting of The Japanese Society for Comparative Physiology and Biochemistry (JSCPB)*, Sapporo, Japan, July, 2014

SUPPLEMENTARY MATERIALS

In the preliminary examination on my initial version of the thesis (23 November, 2016), Prof. Makoto Mizunami (Hokkaido University), Dr. Kazuhiro Wada (Hokkaido University) and Prof. Michael Colombo (University of Otago, New Zealand) gave me a series of instructive comments and suggestions. In response to these major comments, I made revisions on my thesis and added supplementary materials on re-analyses of data. Further minor comments and typos were corrected accordingly in the main text.

- Comment: In Chapter II, experiment 1, please normalize running distance of paired condition by single running, to see if the lesion effect is still significant.

As the dissertation committee required, I normalized the running distance in experiment 1, and the results were shown in **Figure S1**. For the running distance normalized by subtraction (pair/ $\overline{\text{single}}$, **Figure S1-A**), one-way ANOVA detected a significant effect of group ($F_{3,27} = 17.221, P < 0.0001$). Multiple comparisons by Holm's tests revealed significant differences between the Arco vs. sham ($t = 6.148, P < 0.0001$), Arco vs. Nido ($t = 5.287, P = 0.0001$), lateral Arco vs. sham ($t = 4.368, P = 0.0007$) and lateral Arco vs. Nido ($t = 3.722, P = 0.0028$) groups, but not between the sham vs. Nido ($t = 0.302, P = 0.765$) or Arco vs. lateral Arco ($t = 1.358, P = 0.371$) groups. For the running distance normalized by division (pair/ $\overline{\text{single}}$, **Figure S1-B**), a Welch one-way ANOVA detected a significant effect of group ($F_{3,27} = 7.266, P = 0.0033$). Multiple comparisons by Welch's tests (with Holm's corrections) revealed significant differences between the Arco vs. sham ($t = -3.8223, P = 0.0094$), Arco vs. Nido ($t = -3.666, P = 0.0182$), lateral Arco

vs. sham ($t = -3.186, P = 0.0259$) and lateral Arco vs. Nido ($t = -2.969, P = 0.0432$) groups, but not between the sham vs. Nido ($t = -0.451, P = 0.6584$) or Arco vs. lateral Arco ($t = -1.116, P = 0.5768$) groups. According to these reanalysis, I conclude that the Arco and lateral Arco lesion have significant effects on social facilitation of running even after normalization of data.

- Comment: In Chapter II, experiment 1, how was the behavior of the companion chicks?
Was the behavioral effects of lesioned subjects influence the behavior of companion?

As shown in in Chapter II, experiment 1 (Figure II-4A and Figure S1), subject chicks showed suppressed running after Arco lesion and lateral Arco lesion. In order to see if the behavioral changes of the lesioned subjects influenced the behavior of companions that were paired, I calculated the running distance of companions as shown in **Figure S2**. A one-way ANOVA detected a significant effect of group ($F_{3,27} = 7.399, P = 0.0009$). Multiple comparisons by Holm's tests revealed significant differences between the Arco vs. sham ($t = 4.684, P = 0.0004$), but not between Arco vs. Nido ($t = 2.722, P = 0.0561$), and lateral Arco vs. sham ($t = 2.130, P = 0.1697$), the lateral Arco vs. Nido ($t = 0.488, P = 0.6293$) groups, sham vs. Nido ($t = 1.674, P = 0.2113$), or Arco vs. lateral Arco ($t = 2.127, P = 0.1697$) groups. The suppressed running of the subjects with Arco lesion influenced the running of paired intact companion chicks.

In order to see the running distance was correlated between the companions and the subjects, I plotted the running distance as shown in **Figure S3**. A Pearson test revealed a significant positive correlation between the running distance of subject and companion

($r=0.797$, $t=7.111$, $p<0.0001$, $n=31$). It is reasonable to assume that the altered behavior of the subject by lesion influenced the foraging behavior of intact companion chick. I noticed also that the companion chicks tended to run more than the subjects in the sham control, which however does not account for the subject's influence on the companion.

- Comment: In Chapter II, experiment 3, how was the Arco lesion affect the binary choice of chicks?

In Chapter II, experiment 3, we found that the response peck latency was intact after Arco lesion. As required, I analyzed the number of S+ choice and S+ choice ratio to see if Arco lesion affects the choices based on the memorized association between color cues and reward. **Figure S4** showed the choice of S+ in the binary choice. Number of S+ choice was shown in **Figure S4-A**. A logistic regression detected no significant effect of phase ($Z = 0.436, P = 0.6630$) or group ($Z = -0.869, P = 0.3850$). The choice ratio of S+ was shown in **Figure S4-B**. A logistic regression detected no significant effect of phase ($Z = 0.956, P = 0.3390$) or group ($Z = -0.030, P = 0.9760$). I concluded that Arco lesion has no effect on the binary choice of the operated-on subjects, suggesting intact association memory. This result is consistent with that reported previously by Aoki, Csillag and Matsushima (2006).

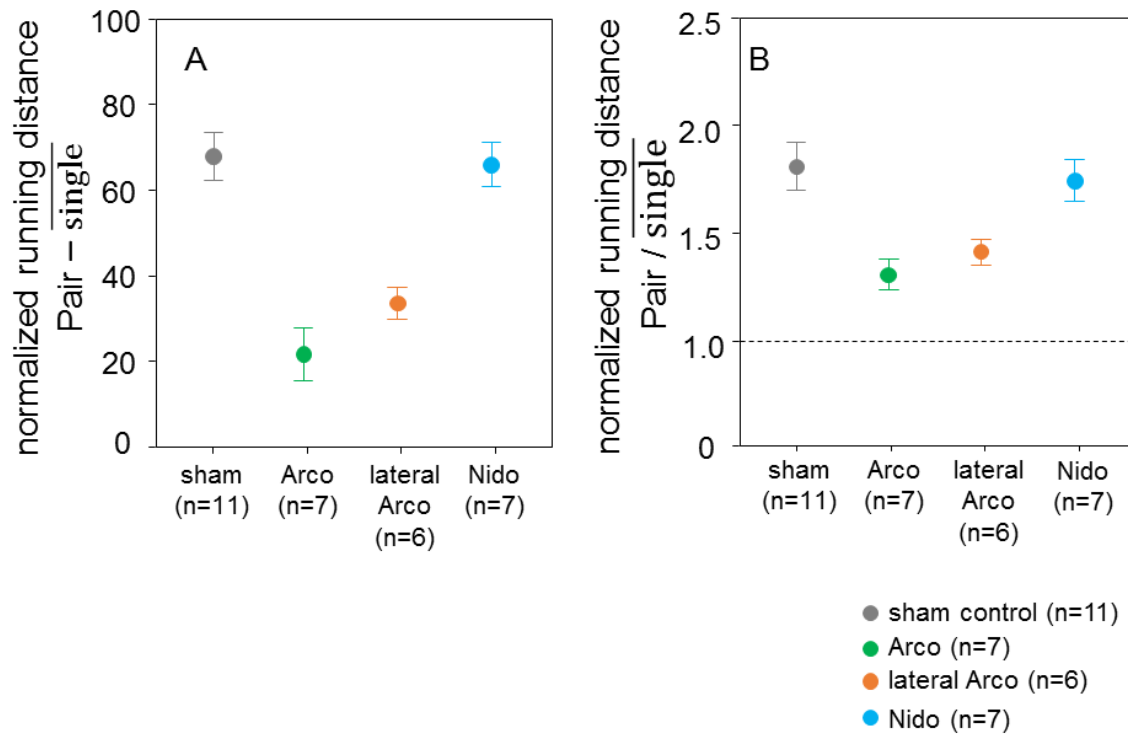


Figure S1. Normalized running distance in the Chapter II, experiment 1. (A) Running distance was normalized by subtraction (pair - single). (B) Running distance was normalized by division (pair / single). Different groups are indicated by different colors (see inset).

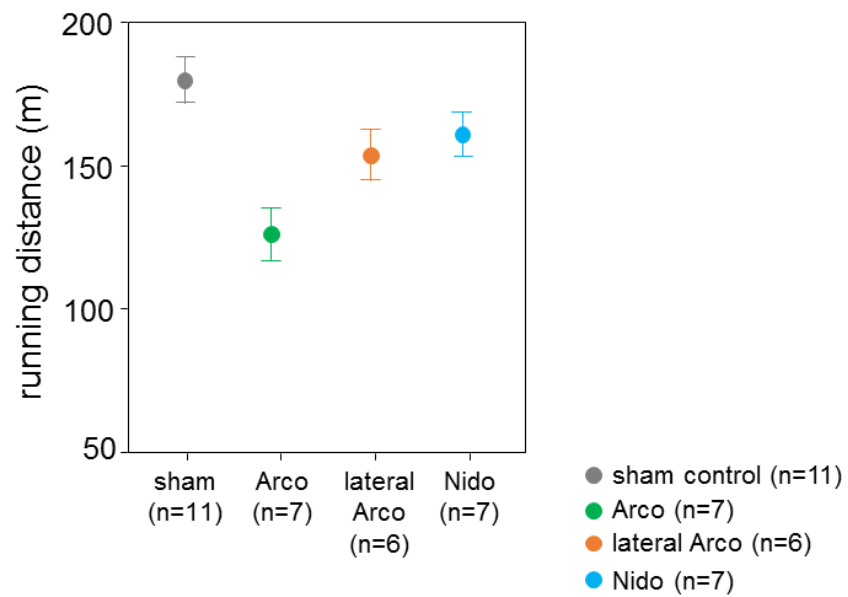


Figure S2. Running distance of companion chick in the pair phase of the Chapter II, experiment 1. Different groups are indicated by different colors (see inset).

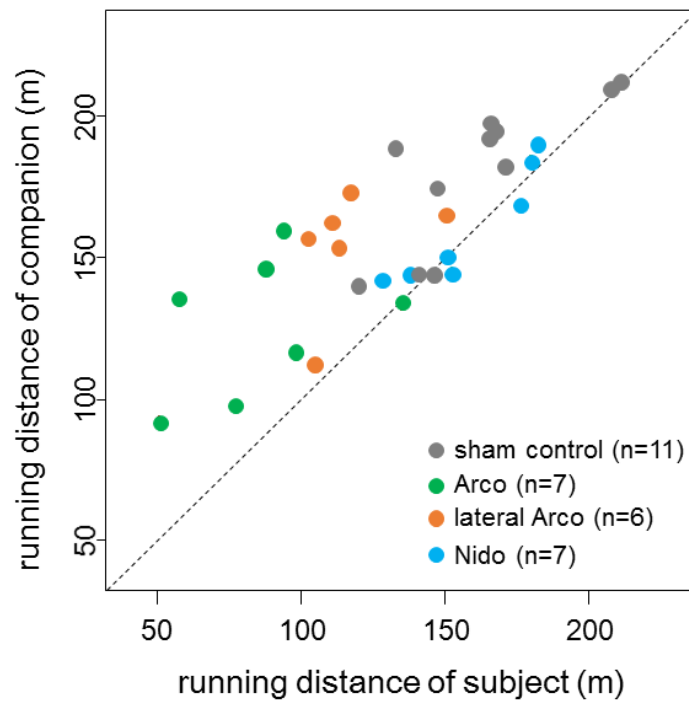


Figure S3. Correlation of the running distance of subject and companion (Chapter II, experiment 1). The running distance of companion was plotted against the running distance of subject. Different groups are indicated by different colors (see inset).

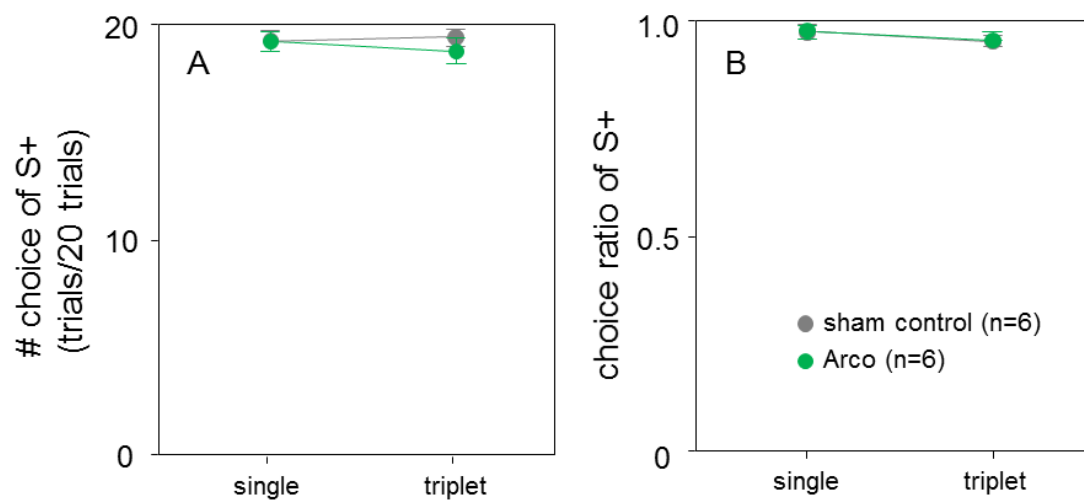


Figure S4. Lesion effect on the choice of S+ in the binary choice of the Chapter II, experiment 3. (A) Number of the choice of S+. **(B)** The choice ratio of S+. Different groups are indicated by different colors (see inset).