学位論文

Study on intraspecific diversity and genetic background of startle response in medaka (*Oryzias latipes*)

（ニホンメダカの驚愕反応の種内多様性と遺伝的要因の解析）

平成２６年１２月博士（理学）申請

東京大学大学院理学系研究科

生物科学専攻

坪子　理美

Contents

Abstract **4**

Abbreviations **6**

General introduction **7**

Chapter 1:

Quantitative comparison of startle response properties among medaka inbred strains . **10**

1) Introduction 11

2) Materials and methods 13

3) Results 17

4) Discussion 20

5) Table and figures 22

Chapter 2:

Analysis of genomic regions related to the startle response properties **30**

1) Introduction 31

2) Materials and methods 33

3) Results 35

4) Discussion 37

5) Table and figures 38

Chapter 3:

Identification of a genomic region responsible for the startle response property **42**

1) Introduction 43

2) Materials and methods 44

3) Results 47

4) Discussion 49

5) Tables and figures.....52

General discussion 64

Ethics statement 68

References 69

Acknowledgements 78

Abstract

Intraspecific behavioral diversity, both within and among populations, is thought to result from genetic polymorphisms. The mechanisms underlying genetic control of behavioral properties, however, remain unclear in wild-type vertebrates, including humans. To explore this issue, I used diverse inbred strains of medaka fish (*Oryzias latipes*) established from the same and different local populations. In the process of inbreeding, the genetic variation within the species had led to differences among strains. The genetic background itself is thought to define strain-specific traits because each inbred strain is genetically homogeneous with a unique genotype.

I established a behavioral paradigm to quantify visually-evoked behavioral characteristics of medaka using a liquid crystal display, and found that extinction of illumination robustly elicits a startle response in medaka. Medaka exhibited a startle response to the visual stimulus by rapidly bending their bodies (C-start) 20-ms after the stimulus presentation. Next, I measured the rate of the response to repeated stimuli (40 times, 1-s interval) among four inbred strains, HNI-I, HNI-II, HO5, and Hd-rR-II1, and quantified two properties of the startle response: sensitivity (response rate to the first stimulus) and attenuation of the response probability with repeated stimulus presentation. Among the four strains, the greatest differences in these properties were detected between HNI-II and Hd-rR-II1. HNI-II exhibited high sensitivity (approximately 80%) and no attenuation, while Hd-rR-II1 exhibited low sensitivity (approximately 50%) and almost complete attenuation after only five stimulus presentations. HNI-II and HNI-I, both of which were generated from the same local-wild population, also showed a difference in attenuation of the response probability. These findings suggested behavioral diversity of the startle response within a local-wild population as well as among different populations.

Linkage analysis with F2 progeny between HNI-II and Hd-rR-II1 revealed different quantitative trait loci (QTL) for sensitivity and attenuation. The QTL for sensitivity was detected on linkage

group (LG) 3 based on analysis of F2 progeny from Hd-rR-II1 females and HNI-II males. The QTL for attenuation was detected with a maximum logarithm of odds (LOD) score of 11.82 on LG 16, based on analysis with the entire F2 population from reciprocal crosses.

To narrow down the candidate region and to examine the interaction with other genomic regions, I established three congenic lines, each of which has a genomic background of Hd-rR-II1 and has only a limited region on chromosome 16 replaced by the HNI-II genome. One of the congenic lines exhibited a significantly lower degree of attenuation than Hd-rR-II1, suggesting that this particular genomic region can alter the behavioral trait without interactions between other loci. The degree of attenuation of the congenic line was still higher than HNI-II, suggesting additive effects of other loci and/or interactive effects with other loci to this trait.

The findings in this study are the first to suggest that a single genomic region is sufficient to generate a certain part of the individual difference in startle behavior between wild-type animal strains. Further identification of genetic polymorphisms that define the behavioral trait will contribute to our understanding of the neural mechanisms underlying behavioral diversity, allowing us to investigate the adaptive significance of intraspecific behavioral polymorphisms of the startle response.

Abbreviations

cM: centiMorgan

LG: linkage group

LOD: logarithm of odds

PCR: polymerase chain reaction

QTL: quantitative trait locus

SEM: standard error of mean

General introduction

Many animals, including humans (Gosling 2001), exhibit intraspecific behavioral diversity in various dimensions, such as startle response, feeding, aggression, and activity in voluntary movement (Réale, et al. 2007, Bouchard and Loehlin 2001, Carter, et al. 2013, Sih, Bell and Johnson 2004, Wilson, et al. 1994). Individual differences in behavioral traits are thought to be an important factor influencing the fitness of each individual depending on the environment and/or a distribution of the phenotype (Smith and Blumstein 2008), and are suggested to be under natural selection (Réale, et al. 2007). In addition, intraspecific behavioral diversity in some animal groups may contribute to the emergence of social organization such as a leader-follower relationship (Webster and Ward 2011, Bergmuller and Taborsky 2010). For example, stickleback individuals exhibiting relatively higher boldness in individual tests tend to lead individuals with lower boldness when foraging as a pair (Harcourt, et al. 2009). In social spiders, it is suggested that individuals with shorter latencies to recover from huddling in air puff tests are tend to participate more in prey capture in a colony (Grinsted, et al. 2013). Also, in mosquito fish, the swimming speed of the fastest fish in a group, observed in individual tests, tends to determine group-level swimming properties (Herbert-Read, et al. 2013). Thus, individual differences in the behavioral characteristics of wild animals have attracted the interest of many researchers, not only in the field of behavioral biology but also in the field of evolutional ecology (Gosling 2001, Réale, et al. 2007, Carter, et al. 2013, Sih, Bell and Johnson 2004). Genetic polymorphisms that determine such behavioral diversity and potentially affect social organization, which could be key factors in the field of evolutionary ecology studies, however, have not yet been well clarified yet.

To explore this issue, I focused on the medaka fish (*Oryzias latipes*), a small teleost with a habit of group forming and coordinated swimming, in my graduate school studies. Over ten inbred strains of medaka of various origins have been generated from over 20 generations of sister-brother mating,

including wild and pet strains (Hyodo-Taguchi, 1996, Spivakov, et al. 2014, Sasado, et al. 2010, Hyodo-Taguchi, 1990, Hyodo-Taguchi, 1983). In the process of inbreeding, the genetic variations that are originally present within the same population distribute to each strain. Because each inbred strain is genetically homogeneous with a unique genotype, the genetic background itself is thought to define strain-specific traits (Kimura, Shimada, et al. 2007, Ishikawa, et al. 1999, Ishikawa, et al. 1999). Therefore, I thought that distinct behavioral traits of the inbred strains could be considered to reflect the behavioral 'personality' of each individual of the original population. I thus expected that analysis of the genetic background of the different behavioral properties of each medaka strain could provide important insight into the genetic background of the behavioral 'personality' of the wild medaka and thus could be an important clue for the further genetic dissection of the behavioral 'personality'.

Importantly, the availability of a dense genetic linkage map (Kimura, Shimada, et al. 2007, Kimura and Naruse, 2010, Kimura, Yoshida, et al. 2005, Naruse, et al. 2000) and large-scale genomic data (Ahsan, et al. 2008, Kasahara, et al. 2007, Kobayashi and Takeda 2008, Takeda 2008) allows us to identify the quantitative trait loci (QTL) for complex traits and to compare genetic polymorphisms in candidate genomic regions among medaka inbred strains. A previous comparative analysis of the genomes of medaka inbred strains revealed a genome-wide single nucleotide polymorphism rate between two inbred strains (HNI-II and Hd-rR-II1) of 3.42%, the highest single nucleotide polymorphism rate observed to date in any vertebrate species (Ahsan, et al. 2008, Kasahara, et al. 2007). This intraspecific genetic variation is considerably large compared with those of other model animal species, such as 0.14% in house mouse (*Mus musculus domesticus*) (Wade, et al. 2002), which originated from limited founder populations, or 0.02% in *Drosophila melanogaster* (Müller, et al. 2012). Medaka is thus considered an ideal animal model for the search of genetic factors that underlie complex traits (Kimura, Shimada, et al. 2007).

To date, although some QTLs for morphologic traits have been identified in medaka by analysis of inbred strains (Kimura, Shimada, et al. 2007, Kimura, Shinya and Naruse, 2012), only one report has described differences in behavioral traits among medaka inbred strains; when humans approach rearing tanks, some inbred strains tend to approach to humans, while other inbred strains tend to dart away from humans (Hyodo-Taguchi, 1990), possibly in response to the human figure. The difference in behavioral traits, however, has not been quantitatively analyzed based on paradigmatic behavioral factors. In the present study, I first quantitatively compared the startle response to extinction of illumination among medaka inbred strains, and then performed a genome-wide QTL analysis to identify genomic regions that are responsible for different behavioral properties.

Chapter 1

Quantitative comparison of startle response properties among medaka inbred strains

Introduction

Escape behaviors and preceding startle responses are essential for animals to avoid danger (Godin 1997, Ruxton, Sherratt and Speed 2004). The execution of such behaviors, however, generally requires a large amount of energy and interferes with other behaviors such as foraging (Quinn and Cresswell 2005). Differences in danger avoidance tendencies among individuals contribute to the emergence of a leader-follower relationship, as observed in some teleost and avian species (Harcourt, et al. 2009, Krause and Ruxton 2002), that is, individuals with high escape tendencies are followed by those with low escape tendencies. Therefore, intraspecific polymorphisms of startle properties could have large effects on the diversity of survival strategies and be under natural selection.

Although previous studies have suggested genetic effects on the startle response polymorphisms, the responsible genes have not yet been identified for the following reasons: 1) Unavailability of inbred strains (van Oers, et al. 2004, Lucas, et al. 2004), and 2) Low reproducibility of observed behavioral differences (Flint, 2003, Flint, Valdar, et al. 2005, Gosling 2001).

To address these problems, I focused on inbred strains of medaka. Studies in our laboratory have established that medaka reproducibly exhibit various social behaviors such as coordinated swimming (schooling) (Imada, et al. 2010), visually-guided group foraging (Ochiai, et al. 2013), and courtship and mate choice (Okuyama, Yokoi, et al. 2014) in experimental setups in the laboratory environment. There is one report to date, however, describing differences in startle response tendencies among medaka inbred strains; some inbred strains tend to approach to humans, while other inbred strains tend to avoid humans (Hyodo-Taguchi, 1990). The difference in behavioral traits, however, has not been analyzed quantitatively.

My observations in the laboratory revealed that medaka exhibit a startle response (quick jump) in response to moving objects, such as humans approaching rearing tanks and hands waved over the tanks. Based on the assumption that this startle response is elicited visually, I attempted to establish a quantitative assay system for the startle response with a visual stimulation setup. In this chapter, I compared startle response properties among medaka inbred strains with this system, aiming to establish a basis for future genetic analyses.

Materials and Methods

(1) Animals

Adult medaka (>3 months after hatching, 2.5-4 cm long) were used for behavioral tests. Four medaka inbred strains used in the experiments, HNI-I, HNI-II, Hd-rR-II1, and HO5 (Table 1) were established and maintained by over 20 generations of full sister-brother mating (Spivakov, et al. 2014, Sasado, et al. 2010, Hyodo-Taguchi, 1990, Hyodo-Taguchi, 1983). The National BioResource Project Medaka provided all of these strains. Water temperature was maintained at approximately 28℃. The light:dark ratio was 14 h:10 h, with lights on from 08:00 to 22:00. Fish were maintained in plastic aquariums (12 cm x 13 cm x 19 cm). Each aquarium was separated by a transparent partition into two ID-tagged compartments, each of which contained two fish. Fish were fed brine shrimp (3 - 4 times daily). Rearing water was circulated among the aquariums, and water loss was replaced with dechlorinated tap water.

(2) Behavioral tests

Behavioral tests were performed over 5 days. On each experimental day, fish were scooped from the aquariums at ~12:00 and placed into ID-tagged plastic cups with rearing water (one pair of fish per cup) for transportation to the observation room. Fish were kept in the observation room for ~3 hours for acclimation. For behavioral observation, a shelf with three transparent glass boards was used (Figure 1A). At ~15:00, each individual fish was placed in a tall Petri dish (8 cm in diameter, filled with water to a 2-3 cm depth, and covered on the side surface with white paper) on the middle shelf with a 4-cm-long plastic plate as a spatial scale. Up to four fish were tested at the same time,

and the other fish were kept under an opaque cover during the trial. After the fish were placed on the middle board, the light in the experiment room was turned off. Visual stimuli were presented with a liquid-crystal display (TSA4634JT, Iiyama, Tokyo, Japan. Response time: 72 ms) set on the upper board of the shelf. Fish were first presented with the light stimulus for 5 min for acclimation, and then for 20s for start-up of the video recording. After the video recording was started, fish were shown a stimulus movie (Figure 1B). Video recording was stopped after the movie ended. Between trials, the light of the room was turned on to change fish. After all trials were complete, the fish were transported back to the rearing room.

(3) Visual stimuli

In the trials, fish were presented a light-dark stimulus movie in which 40 light periods and 40 dark periods appeared alternately. In the experiment shown as "0.5 s" in Figure 8, the dark stimulus was presented every 0.5-s for a period of 1.0-s. In the other experiments, the lengths of the light periods and dark periods were each set to be 1.0-s long as shown in Figure 1B.

(4) Video recording

The time-lapse image of C-start shown in Figure 1E was recorded at 600 frames per second with a digital camera (EXILIM EX-F1, CASIO, Japan). In the experiments shown in Figures 2, 3, 4, 5, 6, 7 and 8, movement of the fish was recorded from underneath at 30 frames per second with a video camera (HDR-HC3, SONY, Japan). The infrared imaging ("night-shot") mode of the video camera was used to observe the behavior of the fish under consecutive light-to-dark transitions.

Infrared LED lights (LB12-WP01, Ebisu-denshi, Osaka, Japan, with a peak wavelength of 850 nm, which is out of the estimated visible wavelength range of medaka (Matsumoto, et al. 2006)) were used for infrared lighting. To illuminate the viewing field uniformly, an opalescent acrylic board (3mm thick) was placed on tall Petri dishes as a light diffuser. To cut off excess visible light from the recorded movies, an infrared filter (IR 78, Fuji film, Japan), which cuts off light with a wavelength <780nm, was set on the lens of the video camera. A pinhole was created at the center of the filter, through which the light-dark changes of the display were recorded (Figure 1C and 1D).

(5) Quantification of response properties for each individual

The startle response was manually scored on the video recordings (30 frames/s), as a quick tail flip with a C-shaped body bend starting within three recording frames after an onset of a light-to-dark transition, with >0.5cm of body moving distance between two adjacent recording frames (corresponding to >15cm/s in velocity). The "sensitivity" was calculated as response probability to the first dark period. The "response stability index" was calculated as the number of dark periods needed before an integrated amount of responses reached half the total amount of response.

In the analysis of sensitivity, a mean value of the 5 experimental days was used as a characteristic index value for each individual. In the analysis of the response stability index, a mean value of 3 days, not including the highest and lowest values over the 5 experimental days, was used for each individual.

(6) Quantification of maximum velocity during a response

The maximum velocity during each response was calculated from fish velocities and time sequences of light-dark changes according to the following procedure using ImageJ

(http://imagej.nih.gov/ij/). First, the recorded movies were imported to ImageJ (Ver.10.2) using QuickTime (Ver. 7.6.4) and each frame of the movies was converted to an 8-bit monochrome image. To calculate the fish velocities, the area inside of the tall Petri dish was defined as the region of interest. The brightness threshold was set to distinguish the fish from the background, and a spatial scale was calibrated using the 4-cm-long plastic plate placed near the tall Petri dish. The positions of the fish were measured using the Particle Analyzer algorithm of ImageJ and the velocities of the fish were calculated at each frame. To track light-dark changes over time, the "Stacks" function of ImageJ was used. The small circular area at the center of the image, representing the light-dark changes recorded through the pinhole of the infrared filter, was selected as a region of interest (Figure 1C and 1D). A time sequence of brightness in the region was obtained by the "Plot Z-axis Profile" command, and the onset of each light-to-dark change was defined as <-1 of transition in brightness from a previous recording frame (in typical cases, mean brightness values in the region were ~85 in light states, and <80 in dark states). Based on the sequential values, the maximum velocity during each response was defined as the highest velocity in three recording frames after a light-to-dark transition.

Results

(1) Behavioral paradigm of visually-evoked startle response

In this study, I newly established a behavioral paradigm to quantify visually-evoked behavioral characteristics of medaka using a liquid crystal display (Figure 1A). Extinction of illumination (color transition from white to black on the entire screen of the liquid crystal display) robustly elicited a transient startle response in medaka fish. At approximately 20-ms after the stimulus presentation, medaka exhibited rapid and transient body bending (Figure 1C and 1D). This startle response resembled the "C-start", a broadly observed escape behavior of teleost fish (Eaton, Lavender and Wieland 1981). Medaka fish responded to the extinction of illumination (light-to-dark stimulus, Figure 1C), but showed no apparent response to dark-to-light stimulus (Figure 1D). I tested four inbred strains from the same and different populations, and detected no prominent difference in either the latency of the response or the motion kinematics (Table 1).

(2) Differences in startle response properties among medaka inbred strains

To investigate changes in the startle response to repeated eliciting stimuli, I used blinking stimuli, where the entire screen flickered 40 times at 1-s intervals (Figure 1B). I examined whether fish exhibited the startle response to each eliciting stimulus based on recorded movies (Figure 1A and 1C). This behavioral test included the same fish everyday for five consecutive days and the startle response rate of each fish was calculated for every stimulus (Figure 2A). Among the four strains, the most prominent differences were detected between HNI-II and Hd-rR-II1 in two features. HNI-II demonstrated the highest sensitivity and no attenuation of the response probability, whereas Hd-rR-II1 demonstrated the lowest sensitivity and almost complete attenuation of the response probability within five repeated stimulus presentations.

Although these two strains showed differences in the response probabilities, neither exhibited a significant decrease in maximum velocity during the response (i.e., response magnitude) and both continued to respond with a velocity of >15 cm/s until they stopped responding (one-way ANOVA; HNI-II: $F(39, 685) = 1.14$, $p > 0.05$; Hd-rR-II1: $F(3, 28) = 2.11$, $p > 0.05$; Figure 5A).

To quantitatively compare the response properties, I defined two indices. The ”sensitivity index” was defined as the probability of a response to the first eliciting stimulus on each of the 5 days. The “response stability index” was defined as the number of eliciting stimuli required before an integrated response reached half the total amount (see Materials and Methods), which negatively correlates with the degree of attenuation. No effect of day on either of the indices was detected (two-way repeated measures ANOVA; sensitivity: $F[4, 280]= 0.31$, $p > 0.05$; response stability index: $F[4, 280] = 1.03$, $p > 0.05$; Figure 4). The sensitivity index differed significantly between HNI-II and Hd-rR-II1 (Scheffe’s F test, $p < 0.01$; Figure 2B and 3A) among the four inbred strains, while the response stability index differed significantly among several combinations (Figure 2C and 3B).

HNI-I and HNI-II showed a difference in attenuation of the startle response probability between them, while both of them originated from the same local-wild population or a deme (Niigata stock) (Hyodo-Taguchi, 1996, Hyodo-Taguchi, 1990, Hyodo-Taguchi, 1983). To investigate whether such behavioral difference also exist within a local-wild population, I examined this behavior in offspring of the original population of HNI-II and HNI-I, maintained as Niigata stock (Hyodo-Taguchi, 1996, Hyodo-Taguchi, 1990). Individuals from Niigata stock had widely ranging response stability index, including one lower than that of any of the HNI-I individuals, to one the same level as that of HNI-II (Figure 6).

(3) Sexual dimorphism in sensitivity in Hd-rR-II1

The sensitivity index was significantly higher in females than in males (two-way ANOVA; sex: $F[1,52] = 9.61$, $p < 0.01$; Figure 7A). This sexual dimorphism was significant only in Hd-rR-II1, although the other inbred strains also showed the same tendency. There was no significant difference in the response stability index between sexes (two-way ANOVA; sex: $F[1,52] = 2.63$, $p = 0.11$; Figure 7B).

(4) Attenuation of the startle response probability with highly frequent stimuli in HNI-II

HNI-II exhibited no attenuation in the response probability to repeated stimulus presentations, suggesting that some mutations in the HNI-II inbred strain resulted in a loss of the ability to exhibit startle response attenuation. To examine whether HNI-II could exhibit startle response attenuation, I presented visual stimuli with a shorter interval, i.e., the display flickered 40 times at 0.5-s intervals. Under these conditions, HNI-II exhibited an attenuated startle response and the response stability index was significantly lower than that when stimuli were presented at 1.0-s intervals (one-way ANOVA; $F[1, 24] = 83.32$, $p < 0.001$; HNI-II, $n = 6$; Hd-rR-II1, $n = 8$; Figure 8C). This finding indicated that HNI-II exhibits an attenuated startle response probability to repeated stimulus presentations. The index in Hd-rR-II1, however, did not differ significantly between the two stimulus presentation conditions (one-way ANOVA; $F[1, 26] = 1.68$, $p > 0.05$; Figure 8C), suggesting that the response stability index of Hd-rR-II1 reached the lowest plateau with stimulus presentation at 1.0-s intervals. There was no significant difference in sensitivity in either HNI-II or Hd-rR-II1 (one-way ANOVA; HNI-II: $F[1, 24] = 0.02$ $p > 0.05$; Hd-rR-II1: $F[1, 26] = 1.82$, $p > 0.05$; Figure 8B). Thus, I concluded that the plasticity of the visual response in HNI-II is not defective.

Discussion

The startle response is a reflexive reaction to potentially stressful environmental stimulation that is observed in most, if not all, animal species (Eaton 1984). Extinction of illumination robustly induced the startle response (C-start) in medaka fish, suggesting that the visual stimulus mimics the sudden appearance of a natural predator above the water (Gwilliam 1963, Russell 1967). Since HNI-II and Hd-rR-II1 were observed to show normal optomotor response (Yuji Suehiro, personal communication, 2009), in which medaka follow a series of black and white stripes, at least both of these inbred strains were supposed to have ability to percept visual stimuli and physically response to visual stimuli. In this study, in addition to HNI-II and Hd-rR-II1, HNI-I and HO5 also showed the startle response to the light-to-dark, not to the dark-to-light, visual stimulus.

The originally established experimental paradigm clearly demonstrated a significant difference in the startle response pattern among medaka inbred strains. The most prominent behavioral differences were detected between HNI-II and Hd-rR-II1, which also have the highest genetic difference based on the base substitution rate (Kasahara, et al. 2007).

In the inter-strain comparison of the response stability index, a difference in behavior was observed between HNI-I and HNI-II, both of which originated from the same local-wild population. Considered along with the varied values of the response stability index observed in the Niigata stock, it is possible that a genetically defined polymorphism of the behavioral property exists in a wild living population. Further genetic characterizations of individuals in the population will help us to determine whether the behavioral differences reflect genetically-affected behavioral diversity in natural environments. As animal personalities can be consistent across different contexts (Ward, et al. 2004, Bell 2005, Réale, et al. 2007, but see Coleman and Wilson 1998), genetic factors that determine the startle response properties may also affect on other behavioral characteristics.

Although HNI-II showed almost no response attenuation to the stimulus presented at 1-s intervals, this strain did exhibit attenuated response probability to the stimuli presented at shorter (0.5-s) intervals. This finding suggests that there are inter-strain differences in the threshold of the frequency of the stimulus to result response inhibition. Also, as the startle response of medaka was all-or-none, which is same as in other teleost species (Medan and Preuss 2014), it is assumed that genetic factors responsible for the behavioral properties modulate the probability of the response arousal in the C-start circuit, which is generally comprises only several neurons (~6 neurons from the retina to the trunk muscles (Medan and Preuss 2014) as shown in Figure 9).

Table 1. Inbred strains and their origins

Strain	Local population	Origin
HNI-II	Northern Japan	Wild population in Niigata city, Niigata Prefecture, Japan
HNI-I		Same wild population as HNI-II
HO5	Southern Japan	Commercial stock purchased in Chiba prefecture, Japan
Hd-rR-II1		Commercial stock purchased in Yatomi, Aichi prefecture, Japan

Figure 1. Experimental design and observed startle response

(A) Experimental apparatus with infrared imaging system. (B) Experimental program. (C) (D) Images from infrared recording. Light-dark changes of the display are represented as the changes in brightness at the center of the image (indicated by white arrows). (C) Response to light-to-dark stimulus. Two individuals (top-right and bottom-left, indicated by magenta arrowheads) exhibit C-start. (D) No apparent response to dark-to-light stimulus. (E) C-start of medaka. Fish bend their body in a “C” shape after a ~20-ms latency.

Figure 2. Comparison of startle response properties among inbred strains

HNI-II (n=20), HNI-I (n=6), HO5 (n=14), and Hd-rR-II1 (n=20). (A) Transition of response probability (average of the 5 experimental days). Bars represent SEM. (B) Sensitivity of four inbred strains. A mean value of the 5 days was used as a characteristic index value for each individual. Bars represent SEM. ** $p<0.01$ by Scheffe's F test. (C) Response stability index of four inbred strains. A mean value of 3 days, not including the highest and lowest values over the 5 days, was used as a characteristic index value for each individual. Bars represent SEM. a, b, c, d, and e indicate $p<0.01$ by Scheffe's F test.

Figure 3. Whole distribution of values of the response properties in the four inbred strains

HNI-II (n=20), HNI-I (n=6), HO5 (n=14), Hd-rR-II1 (n=20). (A) Individual values of sensitivity in four inbred strains. (B) Individual values of response stability index in four inbred strains.

Figure 4. Effect of experimental days on the response properties in the four inbred strains

HNI-II (n=20), HNI-I (n=6), HO5 (n=14), Hd-rR-II1 (n=20). Bars represent SEM. *** $p<0.001$ by two-way repeated measures ANOVA. No significant effect of days, and no significant interaction between day and strain were detected.

Figure 5. Transition of fish velocity

(A) Transition of maximum velocity during startle movement. HNI-II (trials, individuals = 20, 4), Hd-rR-II1 (trials, individuals = 30, 6). Bars represent SEM. In each strain, no significant effect of stimulus number was detected by one-way ANOVA ($p>0.05$). (B) Example of tracked velocity of one HNI-II individual through one trial.

Figure 6. Niigata stock, offspring of the founder population of N1 and N2

N2, N1, and Niigata represent HNI-II, HNI-I, and Niigata stock, respectively. (A) Origins of HNI-II and HNI-I. (B) Individual values of the response stability index of each strain or population.

Figure 7. Sexual dimorphism of the response properties in the four inbred strains

HNI-II (11 males, 9 females), HNI-I (3 males, 3 females), HO5 (6 males, 8 females), Hd-rR-II1 (10 males, 10 females). Bars represent SEM. ** $p<0.01$ and *** $p<0.001$ by two-way ANOVA. No significant effect of sex, and no significant interaction between sex and strain were detected.

Figure 8. Comparison of startle response properties between different stimulus interval conditions

HNI-II (n=6), Hd-rR-II1 (n=8). Bars represent SEM. (A) Transition of response probability to frequent (0.5-s interval) stimulus presentations. (B) Sensitivity in different stimulus interval conditions. Bars represent SEM. In each inbred strain, no significant difference was detected between the 1.0-s and 0.5-s interval conditions, based on one-way ANOVA. (C) Response stability index of two inbred strains. ***$p<0.001$ by one-way ANOVA.

Figure 9. C-start circuit mediated by Mauthner cells in teleost

OT represents optic tectum. Visual input from retina is sent to OT, and signals from OT trigger excitement of a Mauthner cell, the center of C-start. Then the Mauthner cell stimulates trunk muscles on the opposite side of the body through its long axon, causing a dynamic body bend.

Chapter 2

Analysis of genomic regions related to the startle response properties

Introduction

Intraspecific behavioral diversity in startle response may be generated not only by environmental factors, such as learning and development, but also by genetic factors (Réale, et al. 2007, Bouchard and Loehlin 2001). For example, heritability of startle response latency has been demonstrated in an avian species, the great tit (*Parus major*) (van Oers, et al. 2004). Rodents exhibit significant differences between experimental strains in several aspects of the startle response (Glowa and Hansen 1994, Flint, 2003, Flint, Valdar, et al. 2005), suggesting that genetic differences among those strains are related to behavioral diversity. Most of QTL analysis studies with rodent models, however, indicate multiple loci with weak effects, and have not allowed for successful identification of the responsible locus for the differences in the startle response (Flint, 2003, Flint, Valdar, et al. 2005, Mackay, Stone and Ayroles 2009). Inaccuracy and low reproducibility of behavioral phenotyping paradigms also potentially interfere with genetic analyses on behavioral traits (Mackay, Stone and Ayroles 2009, Flint, 2003).

In the first chapter, I demonstrated remarkable differences in the startle behavior properties among inbred strains of medaka. The differences were stable throughout the 5 days of the experimental period, and most prominent between two particular inbred strains, HNI-II and Hd-rR-II1, which have the highest single nucleotide polymorphism rate observed to date in any vertebrate species (Ahsan, et al. 2008, Kasahara, et al. 2007). Importantly, the availability of a dense genetic linkage map (Kimura, Shimada, et al. 2007, Kimura and Naruse, 2010, Kimura, Yoshida, et al. 2005, Naruse, et al. 2000) and large-scale genomic data (Ahsan, et al. 2008, Kasahara, et al. 2007, Kobayashi and Takeda 2008, Takeda 2008) allows us to identify the QTL for complex traits in general and to compare genetic polymorphisms in candidate genomic regions between the medaka inbred strains.

In this chapter, I performed a genome-wide QTL analysis for the two properties of the visually-evoked startle response to search for the genomic region related to the behavioral traits.

Materials and methods

(1) Animals

Adult medaka (>3 months after hatching, 2.5-4 cm long) were used for behavioral tests and genotyping. Two medaka inbred strains, HNI-II and Hd-rR-II1 (Spivakov, et al. 2014, Sasado, et al. 2010, Hyodo-Taguchi, 1990, Hyodo-Taguchi, 1983), were used as founder strains. These strains were established and maintained by over 20 generations of full sister-brother mating, and were provided by the National BioResource Project Medaka. Two F1 hybrids, F1-a (female Hd-rR-II1 x male HNI-II) and F1-b (female HNI-II x male Hd-rR-II1), were used for the experiment shown in Figure 10. The two F2 hybrids used for the QTL analysis, F2-a and F2-b, were obtained from sibling mating within F1-a and F1-b, respectively. The rearing conditions were the same as described in Chapter 1.

(2) Behavioral tests and quantification of the properties

Behavioral tests and quantification of the behavioral properties were performed as described in Chapter 1. The lengths of the light periods (interval of dark stimuli) and dark periods were each set to be 1.0-s long.

(3) Genotyping

For DNA extraction, fish were submerged in ice water (≤ 4 °C) until cessation of gill movement and heartbeat, and then decapitated with a sharp blade. Genomic DNA was extracted using a PI-50 automatic DNA isolation system (Kurabo Industries Ltd., Osaka, Japan) from the decapitated and gutted medaka bodies fixed in 100% ethanol. I used 147 polymerase chain reaction (PCR) product

length polymorphism primers, which were described previously (Kimura, Shinya and Naruse, 2012). PCR and polymorphic analyses were performed as described previously (Kimura and Naruse, 2010, Kimura, Shinya and Naruse, 2012).

(4) QTL analysis

I used a total of 117 F2 individuals for 5 days of behavioral tests. Behavioral tests were performed in 10 divided batches. In each batch, 2 HNI-II (positive control), 2 Hd-rR-II1 (negative control), and 11 or 12 F2 individuals were used. In preliminary experiments, some batches exhibited a low response rate among all individuals, including HNI-II. Therefore, I used the response rate of HNI-II as a criterion for excluding batches with generally low response rates from the dataset for further analysis. I eliminated two batches (23 individuals) in which HNI-II exhibited <70% of the mean response rates throughout the 40 stimulus presentations. I also eliminated four individuals that died after the behavioral tests, and three individuals for which the movie recordings were problematic. As a result, 87 F2 individuals (offspring of F1-a: n=29, offspring of F1-b: n =58) were used for further QTL analysis.

In the analysis of the response stability index, nine individuals with no response were additionally eliminated from the dataset, because I could not define the index in such cases. QTL analysis was performed using R/qtl (Broman, et al. 2003). Nonparametric interval mapping was performed with a “scanone” function, using the argument “model = np”. The LOD threshold for interval mapping (p = 0.05) was determined by 1000 iterated permutations. The bayesian function was used to calculate the Bayes credible interval (95%) for estimation of the QTL location. The fitqtl function was used to calculate the percentage variance explained by the marker nearest the estimated QTL.

Results

(1) Identification of genomic region highly related to attenuation of the startle response probability

To search for candidate genetic regions defining the behavioral diversity of startle response attenuation, I performed a QTL analysis using HNI-II and Hd-rR-II1. I selected the two strains for their most prominent behavioral difference (Figure 2B and 2C), abundance of polymorphic markers (Kimura, Shimada, et al. 2007, Kimura, Yoshida, et al. 2005, Kimura, Shinya and Naruse, 2012), and availability of genome sequence information (Kasahara, et al. 2007) for further investigations. For QTL analysis, I crossed HNI-II and Hd-rR-II1 for two generations and obtained ~100 F2 individuals. The sensitivity and response stability indices were measured in each individual under the 1-s interval stimulus presentation condition. After the behavioral tests, each individual was genotyped with 147 polymorphic markers on the genome, indicated as black bars on the horizontal axes in Figure 10A and 10B, and black arrowheads in Figure 10C, with a marker interval length of ~10-cM.

Analysis of the response stability index detected revealed significant QTL with a maximum LOD score of 11.82 located on LG 16, explaining 26.7% of the variance ($n = 78$, $p < 0.01$; Figure 10C, 10D and Table 2). The response stability index of F1 between HNI-II and Hd-rR-II1 was similar to that of Hd-rR-II1 (Figure 11C), suggesting that recessive genetic factors have a major effect on the behavioral traits of HNI-II.

(2) Analysis of genomic region related to sensitivity

Analysis of the sensitivity index with the entire F2 population revealed no significant QTL ($n = 87$, $p > 0.05$; Figure 10A). In the previous F1 generation, the sensitivity index was lower in progeny

between Hd-rR-II1 females and HNI-II males (F1-a) than in HNI-II, while the index of progeny obtained from the reciprocal cross (F1-b, from HNI-II females and Hd-rR-II1 males) was not significantly lower than in HNI-II (Figure 11B). In the original four inbred strains I used, the sensitivity index of females was significantly higher than that of males (two-way ANOVA; sex: $F_{[1,52]} = 9.61$, $p < 0.01$; Figure 7A), while there was no significant difference in the response stability index between sexes (two-way ANOVA; sex: $F_{[1,52]} = 2.63$, $p = 0.11$; Figure 7B). Thus, the failure to detect a QTL for the sensitivity index might be due to a sexual dimorphism of this behavioral trait. Analysis discriminating F2 populations from different crosses suggested the presence of a QTL that was specifically detected with the offspring of F1-a ($n = 29$, $p < 0.05$; Figure 12A), not with the offspring of F1-b ($n = 58$, $p > 0.05$; Figure 12B), which might support the hypothesis. This QTL was not on LG 1, corresponding to the sex chromosome of medaka (Matsuda, et al. 2007).

Discussion

The genetic polymorphism that determines the difference in the startle response among wild strains based on QTL analysis has not been clarified in rodents (Flint, 2003, Flint, Valdar, et al. 2005, Mackay, Stone and Ayroles 2009). In the present medaka study, QTL analysis suggested a single 30-cM genomic region (18Mbp) for the startle response attenuation, with higher effect (26.7% variance explained) than that of behavioral QTLs previously found in rodent studies (~5% variance explained on average (Flint, 2003)). This result might reflect differences in the complexity of the behavioral modulation system between teleost fish and rodents, and/or the utility of medaka as a model animal for genetic analysis of complex traits. Recently, it has been demonstrated that an inbred mice strain established from a wild population shows unique behavioral and genetic characteristics (Moriwaki, et al. 2009, Kato, et al. 2014, Takahashi, Shiroishi and Koide 2014). Further establishment of inbred strains with various genetic backgrounds may be a breakthrough of behavioral genetics studies with rodents in the future.

For the sensitivity trait, analysis discriminating F2 populations from different crosses suggested the presence of an autosomal QTL that was specifically detected with the offspring of one of the reciprocal crosses. Combined with the sexual dimorphism of this trait in the original strains, this result might support the hypothesis that this autosomal locus has epistatic effects with other loci on sex chromosomes.

The identified genomic region for the response stability index contains approximately 500 predicted genes, and further generation of congenic lines will allow us to narrow the candidate region and to examine epistatic effects between other loci.

Table 2. Location and effect of the QTL detected in the analysis with the whole F2 population

Trait	LG	QTL	LOD score	Nearest marker	Marker location	LOD score	p value	PVE	HNI-II homo	Hetero	Hd-rR-II1 homo
Sensitivity	-	-	-	-	-	-	-	-	-	-	-
Response stability index	16	30 cM	11.82	MID1615	39.75 cM	5.26	$p < 0.001$	26.7	8.9±1.1	3.1±0.7	1.4±1.0

PVE, percent variance explained; HNI-II homo, homozygous for HNI-II alleles; Hetero, heterozygous, Hd-rR-II1 homo, homozygous for Hd-rR-II1 alleles

Figure 10. Quantitative trait analysis with F2 between HNI-II and Hd-rR-II1

In A, B, and C, dashed lines indicate the thresholds (p=0.05) determined by 1000 times of permutation tests. (A) QTL analysis on sensitivity. No significant QTL was detected. (n=87) (B) QTL analysis on response stability index. Significant QTL was detected on chromosome 16. (n=78) (C) QTL analysis on response stability index. The result of chromosome 16 is shown. Black arrowheads indicate marker positions. (D) Genotype-phenotype relationship at a polymorphic marker located at 39.75-cM on LG 16, corresponding to chromosome 16.

Figure 11. Startle response properties of F1 between HNI-II and Hd-rR-II1

F1-a are the progeny of Hd-rR-II1 females and HNI-II males. F1-b are the progeny obtained from an opposite cross, between HNI-II females and Hd-rR-II1 males. HNI-II (n=18), F1-a (n=8), F1-b (n=7) Hd-rR-II1 (n=17) (A) Transition of response probability. Bars represent SEM. (B) Sensitivity of F1-a, F1-b, and their parental strains. Bars represent SEM. *p<0.05 and ** p<0.01 by Scheffe's F test. (C) Response stability index of F1-a, F1-b, and their parental strains. Bars represent SEM. a, b, and c indicate p<0.01 by Scheffe's F test.

Figure 12. Quantitative trait analysis for sensitivity with F2 obtained from different crosses

In A and B, dashed lines indicate the thresholds (p=0.05). (A) QTL analysis with F2 obtained from F1-a, the progeny of Hd-rR-II1 females and HNI-II males. QTL with a maximum LOD score of 4.42 located on LG 3 (explaining 20.9% of the variance) was detected (n=29). (B) QTL analysis with F2 obtained from F1-b, the progeny of HNI-II females and Hd-rR-II1 males. No significant QTL was detected (n=58).

Chapter 3

Identification of a genomic region responsible for the startle response property

Introduction

In most studies using rodent models, behavioral QTLs have a low contribution rate to traits (~5% on average) and complex interactions with other loci (epistasis), making it difficult to identify specific genes or genetic factors responsible for certain traits (Flint, 2003, Flint, Valdar, et al. 2005, Carlborg and Haley 2004). In the previous chapter, I performed a single QTL analysis for the startle response properties in medaka, and successfully detected a genomic region on chromosome 16 highly related to the degree of attenuation of the response probability (26.7% variance explained to the estimate), containing approximately 500 genes. Epistasis with other loci, which could be a major obstacle to further gene identification (Carlborg and Haley 2004) and should be considered in the analysis of gene functions in behavioral regulation, however, had not been examined.

In this chapter, I generated three congenic lines carrying HNI-II homozygous alleles within chromosome 16 and possessing the genetic background of Hd-rR-II1, to examine the effect of each congenic region on the startle response property without epistasis with other loci, as well as to narrow down the candidate region. I compared the values of the response stability index among the obtained congenic lines and founder strains. I also performed pairwise QTL analysis to search for genetic regions that additively affect the trait.

Materials and methods

(1) Animals

Adult medaka (>3 months after hatching, 2.5-4 cm long) were used for mating and behavioral tests. Two medaka inbred strains, HNI-II and Hd-rR-II1 (Spivakov, et al. 2014, Sasado, et al. 2010, Hyodo-Taguchi, 1990, Hyodo-Taguchi, 1983), which are described in Chapters 1 and 2, were used as founder strains for the generation of congenic lines. The Backcross 1 (BC1) generation was generated from a cross between F1 and Hd-rR-II1 and provided by Dr. Minori Shinya at National Institute of Genetics (currently at Keio University). In an establishment process of congenic lines, juvenile medaka (1.5-2.5 months after hatching) were genotyped for selection. Fish were bred at Laboratory of Bioresources, National Institute for Basic Biology (Okazaki, Aichi prefecture) until establishment of each congenic line. The established congenic lines were transported to Room #60 in Faculty of Science Building #2 or Biotron Room #2, Department of Biological Sciences, Graduate School of Science, the University of Tokyo (Bunkyo-ku, Tokyo) and then used for behavioral tests.

(2) Genotyping

For DNA extraction, fish were submerged in ice water (≤ 4 °C) until cessation of fin and trunk movement, which usually occurred in several seconds. The anesthetized fish were quickly moved onto a paper towel soaked in rearing water. After having 4 to 10 mm^2 of tail fin cut off with ophthalmologic scissors, each fish was individually moved to a rearing tank with an ID label. Genomic DNA was extracted using KAPA Express Extract (Kapa Biosystems, Inc., MA, USA) from each tail fin fixed in 100% ethanol. A total of 187 sets of PCR product length polymorphism primers (spaced at roughly 5-15 cM of intervals) for the whole genome were designed and provided by Dr.

Shinya (Table 3). PCR and polymorphic analyses were performed following the method of Kimura *et al*. (Kimura, Yoshida, et al. 2005).

(3) Backcrossing and incrossing

The speed congenic method (Wong 2002) was used for efficient establishment of congenic lines (Figure 13). In the backcrossing and incrossing processes, replacement rate, i.e., percentage of Hd-rR-II1 alleles in a whole genome except for LG16, was calculated for each obtained individual at each generation. Individuals with relatively higher replacement rates (in most cases, individuals that ranked within the top three) and carrying HNI-II heterozygous alleles within LG16 were selected for backcrossing or incrossing to obtain the next generation. Consecutive backcrossing was continued until the replacement rate reached ~99%, and then incrossing was performed between selected individuals.

(4) Behavioral tests and quantification of the properties

Behavioral tests and quantification of the behavioral properties were performed as that described in Chapter 1. The lengths of the light periods (interval of dark stimuli) and dark periods were each set to be 1.0-s long.

(5) Pairwise QTL analysis for detection of additive effects

The same genotype-phenotype dataset as one described in Chapter 2 was used for QTL analysis. Pairwise QTL analysis was performed using R/qtl (Broman, et al. 2003) with a “scantwo” function. The LOD threshold for interval mapping ($p = 0.05$) was determined by 100 iterated

permutations. The bayesian function was used to calculate the Bayes credible interval (95%) for estimation of the QTL location. The fitqtl function was used to calculate the percentage variance explained by the marker nearest the estimated QTL.

Results

(1) Generation of congenic lines

I generated three congenic strains from HNI-II and Hd-rR-II1, a donor strain and a recipient strain, respectively (Figures 13 and 14). First, I selected an individual with the highest replacement rate (83.0%) from the BC1 generation. Then I performed consecutive selective backcrossing for 4 generations to increase replacement rates and create lineages with different congenic regions by stochastic chromosomal crossovers (Figure 13 and Table 4). Finally, I established three different congenic lines by incrossing, making each congenic region (~30cM, roughly equivalent to 15Mb) homozygous for HNI-II alleles (Figure 13, and Table 4 and 5). The congenic regions of the three lines partially overlapped each other (Table 4 and 5). The replacement rates of the resulting congenic lines were 98.7% to 99.2% (Table 5). It took ~1.5 years from the first backcrossing (BC1 x Hd-rR-II1) to obtainment of the second generation of the congenic lines (BC4F2).

(2) Comparison of response stability index with founder strains

To narrow down the candidate region on chromosome 16, and to examine independent effects of each region without epistasis, I compared values of the response stability index among the established congenic lines and the founder strains. Among the three congenic lines, lines A, B and C, only line C had a significantly higher response stability index than the recipient strain, Hd-rR-II1 (Scheffe's F test, $p < 0.01$; Figure 14). This finding suggests that the HNI-II homozygous region specific to line C contributes to the behavioral change without interactions with other loci. All three congenic lines, including line C, had significantly lower response stability index value than HNI-II, suggesting the presence of additional loci that have additive and/or epistatic effects on this trait.

(3) Analysis of additive and interactive QTL effects

To search for additional regions responsible to for the response stability index and to estimate interactions between genomic regions for the trait, I performed a pairwise QTL analysis (Broman, et al. 2003), which allows for the detection of pairs of QTLs that significantly affecting a trait of interest (Broman, et al. 2003, Togawa, et al. 2006). There were mainly two different analysis models available in the R/qtl package for pairwise QTL analysis; one for detecting pairs of additive QTLs, i.e. QTLs independently affecting the certain trait without epistasis, and one for examination of epistatic interactions (Broman, et al. 2003).

Comparison between additive QTL models and single QTL models, which detects pairs of additive QTLs assuming no epistasis, indicated an additive QTL on LG 21 ($n = 78$, $p < 0.05$; Figure 15 and 16), in addition to the one on LG 16 that was previously detected in the single QTL analysis described in Chapter 2. The analysis showed no other significant pair of additive QTLs for the trait (Figure 15).

Comparison between the full QTL model and the additive QTL model, which suggests epistasis between two QTLs, detected multiple pairs of loci across the whole genome ($n = 78$, $p < 0.05$; Figure 15).

Discussion

One of the established congenic lines had a significantly higher response stability index than the background Hd-rR-II1 strain, suggesting that the HNI-II homozygous region specific to this line is sufficient to generate the behavioral change independent from other loci. The congenic region specific to this line roughly corresponds to 7Mbp on chromosome 16, containing approximately 180 genes. The value of the response stability index was ~4 in the congenic line, and ~20 and ~1 in HNI-II and Hd-rR-II1, respectively. This value in the congenic line is reasonable as the estimated effect of the QTL was ~27% in the F2 population obtained from HNI-II and Hd-rR-II1. The candidate region contains genes related to neuronal excitability and/or neural development (e.g. PRDC13: PR domain containing 3; KCNS2: K^+ voltage-gated channel, delayed-rectifier, subfamily S, member 2; ADCY3: adenylate cyclase 3), and modulation of gene translation (e.g. ARID1A: AT rich interactive domain 1A; PNISR: PNN-interacting serine/arginine-rich), all of which show base substitutions and/or insertion/deletion between HNI-II and Hd-rR-II1 (Table 6). These genes could be preferential targets for further search by expression analysis, transgenesis and/or genome editing, although >100 of the other genes also hold the potential as responsible factors. Genetic comparison with HNI-I and HO5, which showed the intermediate phenotypes, would also help us to identify the responsible genes.

The pairwise QTL analysis indicated another candidate region affecting this trait on a different chromosome. Because this second candidate region on LG 21 is suggested to act independently from the region previously identified on LG 16, it may be possible to examine practical effects and behavioral regulation mechanisms for each individual region. For example, behavioral tests of a chromosome 16 consomic line and a chromosome 21 consomic line, one of which was recently established by Dr. Shinya (personal communication), will allow us to examine independent effects

of both of these chromosomes, excluding effects of the other regions. Gene duplications or the existence of genes with similar functions, which could be found in a homology search or gene ontology search, might explain the genetic background of the additive effects detected between the two chromosomes.

Through the establishment process of the congenic lines, there were two major obstacles in backcrossing and incrossing. One is low emergence rate of individuals with expected genotypes, which is inherent to the method relying on stochastic chromosomal crossovers. In BC4F1 generation of the lineage for establishment of line C, only one male and one female, out of 20 males and 35 females, respectively, were obtained with the expected genotype (Table 4). The other problem was sex conversions, most of which were from female to male (Tables 4, indicated as “white male”). It was initially observed in the fish of BC4F1 generation in February 2014 at Laboratory of Bioresources, National Institute for Basic Biology (Table 4), and frequently occurred in establishment and maintenance of the congenic lines. For example, 5 out of 7 individuals with white body color, which supposed be females under normal conditions, showed male-type morphology of their fins and infertility as females. Some of such “white males” succeeded in mating with females. This phenomenon was not attributed to specific regions on chromosome 16, since it occurred in all of the three lineages for the congenic lines, and also in their siblings with Hd-rR-II1 homozygous genotypes at all of the polymorphic markers. As similar problem also occurred in other strains kept in the same rearing room, it was suspected that sex differentiation was disturbed in the breeding environment, possibly because of high water temperature at embryonic stages (Sato, et al. 2005).

Morphologic and electrophysiologic characteristics of the neural circuit of the startle response (C-start) have been well studied in various teleost species, clarifying the simple composition of only several neural components from a sensory organ to motor neurons (Medan and Preuss 2014, also see

Figure 9). Gene expression analysis focused on those neurons will help us to understand the mechanisms of developmental and functional regulation of the C-start circuit, and provide further insight into the molecular background of the behavioral polymorphisms in this response.

Table 3. Positions of polymorphic markers for marker-assisted selection in generation of congenic lines

LG	Genetic distance (cM)
1	0
	11.9
	20.5
	32.8
	43.2
	48.8
	57.8
	67.1
	72.4
2	0
	5
	16.8
	23.1
	32.4
	32.4
	39.3
	54
3	0
	8.5
	18.1
	27.4
	38.9
	47.8
	56.3
	66.9
4	0
	12.7
	25.4
	29.4
	38.7
	48.1
	57.8
5	0
	10.1
	20.9
	30.6
	37.5
	46.1
	55.7
	61.9
6	0
	8.9
	16.1
	21.1
	33.7
	43.6
	52.2

LG	Genetic distance (cM)
7	0
	12.5
	22.6
	31.3
	41
	46.9
	54.2
8	0
	9
	18
	25.6
	34.2
	39.8
	52.7
	58.7
9	0
	4.7
	17.6
	29.3
	36.5
	46.2
	56.6
10	0
	9.3
	20.1
	29.8
	41.8
	50.1
	62.8
11	0
	2.6
	15.6
	26
	31.3
	39.2
	51.5
	57.7
12	0
	9.7
	21.7
	27
	33.6
	46.2
	50.9
	59.8

LG	Genetic distance (cM)
13	0
	11.1
	22.9
	32.9
	44
	56.8
	68.9
14	0
	7.6
	20.3
	25.3
	33.6
	42.6
	54.5
	57.3
15	0
	9.8
	21.1
	29.1
	38.1
	46.4
	53.7
16	0
	10.9
	16.5
	24.8
	36.4
	46.2
	54.2
	60.1
17	0
	6.6
	18.5
	21.9
	31.6
	38.8
	48.8
	57.1
	66.8
18	0
	12.7
	19.3
	25.3
	36.2
	45.9
	52.2
	59.8
	70

LG	Genetic distance (cM)
19	0
	5.3
	12.6
	20.6
	29.6
	38.4
	47.6
	55.9
20	0
	4.7
	14
	20.9
	29.9
	38.2
	48.6
	56.2
21	0
	11.2
	20.8
	25.9
	34.2
	44.7
	50.3
	60.8
22	0
	7.3
	14.2
	21.8
	28.7
	39.2
	48.6
	54.9
23	0
	12.7
	17.7
	29
	39.1
	48.1
	60.1
24	0
	5.6
	16.3
	18.9
	30.1
	39.1
	46.4
	53.6

Table 4. Genotypes of individuals obtained by consecutive selective backcrossing and incrossing

A

LG 16 Expected genotype								Name of individual	Note	Body color	Selection	Generation
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_17		yellow		BC4F1
R	R	R	R	R	R	R	R	LG16_BC4_p2-2_18		yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p2-2_32	Yellow female	yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p2-2_33		yellow		
?	?	?	R	R	R	R	R	LG16_BC4_p2-2_1		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_3		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_5		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_7		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_9		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_16		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_21		yellow		
H	H	H	H	R	R	R	R	LG16_BC4_p2-2_34		yellow		
H	?	H	H	R	R	R	R	LG16_BC4_p2-2_19		yellow		
H	?	N	?	R	R	R	R	LG16_BC4_p2-2_22		yellow		
N	H	N	N	R	R	R	R	LG16_BC4_p2-2_20		yellow		
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_11		yellow	†	
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_13		yellow	*	
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_15		yellow		
R	R	R	H	R	R	R	R	LG16_BC4_p2-2_4	White male	white		
R	R	R	?	H	H	R	R	LG16_BC4_p2-2_6	White male	white		
R	R	R	H	?	H	R	R	LG16_BC4_p2-2_25		white		
R	R	R	R	H	H	R	R	LG16_BC4_p2-2_31		white		
H	H	H	H	H	H	R	R	LG16_BC4_p2-2_28		white		
H	H	H	H	H	H	R	R	LG16_BC4_p2-2_8		white		
H	H	H	H	H	H	R	R	LG16_BC4_p2-2_10		white		
H	H	H	H	H	H	R	R	LG16_BC4_p2-2_14		white		
H	H	H	H	H	H	R	R	LG16_BC4_p2-2_26		white		
H	H	H	H	H	H	R	R	LG16_BC4_p2-2_27		white		
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_2		white	*	
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_12		white		
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_24		white	†	
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_29		white		
N	N	N	N	R	R	R	R	LG16_BC4_p2-2_30	White male	white		

B

LG 16 Expected genotype								Name of individual	Note	Body color	Selection	Generation
R	R	R	R	R	R	R	R	LG16_BC4_p6_5		yellow		BC4F1
R	R	R	R	R	R	R	R	LG16_BC4_p6_14		yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p6_18		yellow		
N	N	N	N	H	H	H	R	LG16_BC4_p6_9		yellow		
N	N	N	N	H	H	R	R	LG16_BC4_p6_11		yellow		
N	N	N	N	H	H	R	R	LG16_BC4_p6_1		yellow		
N	N	N	N	H	H	R	R	LG16_BC4_p6_3		yellow		
N	N	N	N	H	H	R	R	LG16_BC4_p6_13		yellow		
?	N	N	N	H	H	R	R	LG16_BC4_p6_7		yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p6_10	Dead	yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p6_12		yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p6_15	Infertile	yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p6_16	Dead	yellow		
R	R	R	R	R	R	R	R	LG16_BC4_p6_19		yellow	*	
R	R	R	R	R	R	R	R	LG16_BC4_p6_21	White male	white		
R	R	R	R	R	R	R	R	LG16_BC4_p6_20		white		
H	H	H	H	R	R	R	R	LG16_BC4_p6_2		white		
H	H	H	H	R	R	R	R	LG16_BC4_p6_4		white		
H	H	H	H	R	R	R	R	LG16_BC4_p6_8		white		
N	N	N	N	R	R	R	R	LG16_BC4_p6_12		white		
N	N	N	N	R	R	R	R	LG16_BC4_p6_17		white	*	
N	N	N	N	R	R	R	R	LG16_BC4_p6_22	White male	white		
?	?	?	?	R	R	R	R	LG16_BC4_p6_6		white		

Table 4 (continued)

C

| LG 16 | | |

Table 5. Congenic regions and replacement rates of established congenic lines

LG	Marker ID	Genetic distance (cM)	Line A	Line B	Line C
16	G040S646	0	N2	R	R
	G013S76	10.9	N2	R	R
	G061S209	16.5	N2	N2	R
	OL04A02	24.8	N2	N2	R
	OL01D05	36.4	R	N2	N2
	OL00E04	46.2	R	N2	N2
	OL04D07	54.2	R	R	N2
	OL03C04	60.1	R	R	N2
The other LGs (Replacement rate)			R / Hetero (98.7%)	R / Hetero (99.2%)	R / Hetero (98.9%)

N2, homozygous for HNI-II alleles; R, homozygous for Hd-rR-II1 alleles; Hetero, heterozygous; Shaded areas represent the congenic regions.

Table 6. Genes with base substitutions and/or insertions/deletions between HNI-II and Hd-rR-II1 in the congenic region specific to line C

Non-synonymous change in a.a.		gene	description
a.a. position	a.a. change		
		FAM46A (1 of 2)	family with sequence similarity 46, member A [Source:HGNC Symbol;Acc:18345]
1122	P/R	IBTK	inhibitor of Bruton agammaglobulinemia tyrosine kinase [Source:HGNC Symbol;Acc:17853]
		TPBG	trophoblast glycoprotein [Source:HGNC Symbol;Acc:12004]
244	D/E	UBE2CBP	ubiquitin-conjugating enzyme E2C binding protein [Source:HGNC Symbol;Acc:21381]
		DOPEY1	dopey family member 1 [Source:HGNC Symbol;Acc:21194]
		SNAP91 (1 of 2)	synaptosomal-associated protein, 91kDa homolog (mouse) [Source:HGNC Symbol;Acc:14986]
		PRSS35 (1 of 2)	protease, serine, 35 [Source:HGNC Symbol;Acc:21387]
70	A/T	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic [Source:HGNC Symbol;Acc:6983]
188	R/K	PRDM13	PR domain containing 13 [Source:HGNC Symbol;Acc:13998]
		USP45	ubiquitin specific peptidase 45 [Source:HGNC Symbol;Acc:20080]
592	R/C	PNISR	PNN-interacting serine/arginine-rich protein [Source:HGNC Symbol;Acc:21222]
		C6orf168(1 of 2)	chromosome 6 open reading frame 168 [Source:HGNC Symbol;Acc:20742]
		FBXL4	F-box and leucine-rich repeat protein 4 [Source:HGNC Symbol;Acc:13601]
		POU3F2	POU class 3 homeobox 2 [Source:HGNC Symbol;Acc:9215]
440	A/G	MMS22L	MMS22-like, DNA repair protein [Source:HGNC Symbol;Acc:21475]
		KLHL32	kelch-like 32 (Drosophila) [Source:HGNC Symbol;Acc:21221]
564	S/N	FZD6	frizzled family receptor 6 [Source:HGNC Symbol;Acc:4044]
7	R/P	CTHRC1	collagen triple helix repeat containing 1 [Source:HGNC Symbol;Acc:18831]
		SLC25A32	solute carrier family 25, member 32 [Source:HGNC Symbol;Acc:29683]
		DCAF13	DDB1 and CUL4 associated factor 13 [Source:HGNC Symbol;Acc:24535]
		RIMS2 (1 of 2)	regulating synaptic membrane exocytosis 2 [Source:HGNC Symbol;Acc:17283]
		TM7SF4	transmembrane 7 superfamily member 4 [Source:HGNC Symbol;Acc:18549]
		DPYS	dihydropyrimidinase [Source:HGNC Symbol;Acc:3013]
		ADCY3	adenylate cyclase 3 [Source:HGNC Symbol;Acc:234]
		WDR26 (2 of 2)	WD repeat domain 26 [Source:HGNC Symbol;Acc:21208]
		ZUFSP	zinc finger with UFM1-specific peptidase domain [Source:HGNC Symbol;Acc:21224]
		GPRC6A (1 of 2)	G protein-coupled receptor, family C, group 6, member A [Source:HGNC Symbol;Acc:18510]
		PTPN2 (1 of 2)	protein tyrosine phosphatase, non-receptor type 2 [Source:HGNC Symbol;Acc:9650]
		CEP76	centrosomal protein 76kDa [Source:HGNC Symbol;Acc:25727]
		ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein [Source:HGNC Symbol;Acc:5363]
		NCALD (1 of 2)	neurocalcin delta [Source:HGNC Symbol;Acc:7655]
		GRHL2 (2 of 2)	grainyhead-like 2 (Drosophila) [Source:HGNC Symbol;Acc:2799]
		ZNF706 (2 of 2)	zinc finger protein 706 [Source:HGNC Symbol;Acc:24992]
		RNF19A (2 of 2)	ring finger protein 19A [Source:HGNC Symbol;Acc:13432]
		SPAG1 (1 of 2)	sperm associated antigen 1 [Source:HGNC Symbol;Acc:11212]
		FBXO43	F-box protein 43 [Source:HGNC Symbol;Acc:28521]
		RGS22	regulator of G-protein signaling 22 [Source:HGNC Symbol;Acc:24499]
		VPS13B (4 of 4)	vacuolar protein sorting 13 homolog B (yeast) [Source:HGNC Symbol;Acc:2183]
		VPS13B (3 of 4)	vacuolar protein sorting 13 homolog B (yeast) [Source:HGNC Symbol;Acc:2183]

Table 6 (continued 1)

Non-synonymous change in a.a.		gene	description
a.a. position	a.a. change		
		VPS13B (1 of 4)	vacuolar protein sorting 13 homolog B (yeast) [Source:HGNC Symbol;Acc:2183]
		VPS13B (2 of 4)	vacuolar protein sorting 13 homolog B (yeast) [Source:HGNC Symbol;Acc:2183]
		OSR2	odd-skipped related 2 (Drosophila) [Source:HGNC Symbol;Acc:15830]
		KCNS2	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 2 [Source:HGNC Symbol;Acc:6301]
		NIPAL2	NIPA-like domain containing 2 [Source:HGNC Symbol;Acc:25854]
308	D/G	POP1	processing of precursor 1, ribonuclease P/MRP subunit (S. cerevisiae) [Source:HGNC Symbol;Acc:30129]
		HRSP12	heat-responsive protein 12 [Source:HGNC Symbol;Acc:16897]
376	D/N	MATN2	matrilin 2 [Source:HGNC Symbol;Acc:6908]
287	D/N	MATN2	
335	D/N	MATN2	
330	F/Y	MATN2	
241	F/Y	MATN2	
177	D/H	LAPTM4B (1 of 2)	lysosomal protein transmembrane 4 beta [Source:HGNC Symbol;Acc:13646]
		MTDH	metadherin [Source:HGNC Symbol;Acc:29608]
		UBR5	ubiquitin protein ligase E3 component n-recognin 5 [Source:HGNC Symbol;Acc:16806]
		KLF10	Kruppel-like factor 10 [Source:HGNC Symbol;Acc:11810]
		AZIN1 (1 of 2)	antizyme inhibitor 1 [Source:HGNC Symbol;Acc:16432]
		ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1 [Source:HGNC Symbol;Acc:856]
40	A/T	SPIRE1 (2 of 2)	spire homolog 1 (Drosophila) [Source:HGNC Symbol;Acc:30622]
69	S/F	SPIRE1 (2 of 2)	
		RNF144B	ring finger protein 144B [Source:HGNC Symbol;Acc:21578]
567	L/V	SQLE	squalene epoxidase [Source:HGNC Symbol;Acc:11279]
535	L/V	SQLE	
		OXR1	oxidation resistance 1 [Source:HGNC Symbol;Acc:15822]
		ZFPM2	zinc finger protein, multitype 2 [Source:HGNC Symbol;Acc:16700]
		5S_rRNA	5S ribosomal RNA [Source:RFAM;Acc:RF00001]
		SEH1L	SEH1-like (S. cerevisiae) [Source:HGNC Symbol;Acc:30379]
		CEP192	centrosomal protein 192kDa [Source:HGNC Symbol;Acc:25515]
		C18orf1	chromosome 18 open reading frame 1 [Source:HGNC Symbol;Acc:1224]
		FAM210A	family with sequence similarity 210, member A [Source:HGNC Symbol;Acc:28346]
311	V/M	PREB	prolactin regulatory element binding [Source:HGNC Symbol;Acc:9356]
29	P/L	RBM42	RNA binding motif protein 42 [Source:HGNC Symbol;Acc:28117]
		FBL	fibrillarin [Source:HGNC Symbol;Acc:3599]
		DYRK1B	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B [Source:HGNC Symbol;Acc:3092]
388	E/D	PPOX	protoporphyrinogen oxidase [Source:HGNC Symbol;Acc:9280]
400	C/Y	PPOX	

Table 6 (continued 2)

Non-synonymous change in a.a.		gene	description
a.a. position	a.a. change		
458	F/L	SLC6A20	solute carrier family 6 (proline IMINO transporter), member 20 [Source:HGNC Symbol;Acc:30927]
194	T/S	SLC6A20	
158	R/W	SLC6A20	
		C3orf23	chromosome 3 open reading frame 23 [Source:HGNC Symbol;Acc:25241]
		RRP15	ribosomal RNA processing 15 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:24255]
		5S_rRNA	5S ribosomal RNA [Source:RFAM;Acc:RF00001]
		CNOT10	CCR4-NOT transcription complex, subunit 10 [Source:HGNC Symbol;Acc:23817]
		ANO10	anoctamin 10 [Source:HGNC Symbol;Acc:25519]
		SNRK	SNF related kinase [Source:HGNC Symbol;Acc:30598]
		ABHD5 (2 of 2)	abhydrolase domain containing 5 [Source:HGNC Symbol;Acc:21396]
		CNDP1	carnosine dipeptidase 1 (metallopeptidase M20 family) [Source:HGNC Symbol;Acc:20675]
320	Q/E	BAI1 (2 of 2)	brain-specific angiogenesis inhibitor 1 [Source:HGNC Symbol;Acc:943]
283	S/A	x	x
124	V/L	x	x
		NOD1	nucleotide-binding oligomerization domain containing 1 [Source:HGNC Symbol;Acc:16390]
282	L/V	ENPP2 (1 of 2)	ectonucleotide pyrophosphatase/phosphodiesterase 2 [Source:HGNC Symbol;Acc:3357]
278	L/V	ENPP2 (1 of 2)	
38	I/M	x	x
		TAF2	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150kDa [Source:HGNC Symbol;Acc:11536]
5	L/F	x	x
		Q05KA0_ORYLA	Myosin heavy chain [Source:UniProtKB/TrEMBL;Acc:Q05KA0]
		HACE1	HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1 [Source:HGNC Symbol;Acc:21033]
		KIAA1009 (2 of 2)	KIAA1009 [Source:HGNC Symbol;Acc:21107]
		C3orf39	chromosome 3 open reading frame 39 [Source:HGNC Symbol;Acc:25902]
		CNIH4	cornichon homolog 4 (Drosophila) [Source:HGNC Symbol;Acc:25013]
88	I/M	CYB5R4	cytochrome b5 reductase 4 [Source:HGNC Symbol;Acc:20147]
1045	A/T	ASCC3	activating signal cointegrator 1 complex subunit 3 [Source:HGNC Symbol;Acc:18697]
		GADL1	glutamate decarboxylase-like 1 [Source:HGNC Symbol;Acc:27949]
		STT3B (2 of 2)	STT3, subunit of the oligosaccharyltransferase complex, homolog B (S. cerevisiae) [Source:HGNC Symbol;Acc:30611]
		KIAA1143	KIAA1143 [Source:HGNC Symbol;Acc:29198]
		ZDHHC3	zinc finger, DHHC-type containing 3 [Source:HGNC Symbol;Acc:18470]
		MC2R	melanocortin 2 receptor (adrenocorticotropic hormone) [Source:HGNC Symbol;Acc:6930]
		MC5R	melanocortin 5 receptor [Source:HGNC Symbol;Acc:6933]
		TCEA3	transcription elongation factor A (SII), 3 [Source:HGNC Symbol;Acc:11615]
		ZDHHC18 (1 of 2)	zinc finger, DHHC-type containing 18 [Source:HGNC Symbol;Acc:20712]
255	F/Y	PIGV	phosphatidylinositol glycan anchor biosynthesis, class V [Source:HGNC Symbol;Acc:26031]
		ARID1A	AT rich interactive domain 1A (SWI-like) [Source:HGNC Symbol;Acc:11110]

Table 6 (continued 3)

Non-synonymous change in a.a.		gene	description
a.a. position	a.a. change		
289	V/L	GPN2	GPN-loop GTPase 2 [Source:HGNC Symbol;Acc:25513]
478	T/M	GPATCH3	G patch domain containing 3 [Source:HGNC Symbol;Acc:25720]
280	D/E	GPATCH3	
259	W/L	GPATCH3	
		NR0B2	nuclear receptor subfamily 0, group B, member 2 [Source:HGNC Symbol;Acc:7961]
183	R/H	C1orf172	chromosome 1 open reading frame 172 [Source:HGNC Symbol;Acc:26624]
		TMEM222	transmembrane protein 222 [Source:HGNC Symbol;Acc:25363]
		WDTC1	WD and tetratricopeptide repeats 1 [Source:HGNC Symbol;Acc:29175]
240	G/S	CEP85	centrosomal protein 85kDa [Source:HGNC Symbol;Acc:25309]
		FEZ2 (2 of 2)	fasciculation and elongation protein zeta 2 (zygin II) [Source:HGNC Symbol;Acc:3660]
		CLEC3B (2 of 2)	C-type lectin domain family 3, member B [Source:HGNC Symbol;Acc:11891]
		TMEM158	transmembrane protein 158 (gene/pseudogene) [Source:HGNC Symbol;Acc:30293]
529	G/C	LARS2	leucyl-tRNA synthetase 2, mitochondrial [Source:HGNC Symbol;Acc:17095]
		LIMD1 (1 of 2)	LIM domains containing 1 [Source:HGNC Symbol;Acc:6612]
		DEPTOR	DEP domain containing MTOR-interacting protein [Source:HGNC Symbol;Acc:22953]
		COL14A1 (2 of 2)	collagen, type XIV, alpha 1 [Source:HGNC Symbol;Acc:2191]
		MRPL13	mitochondrial ribosomal protein L13 [Source:HGNC Symbol;Acc:14278]
		DSCC1	defective in sister chromatid cohesion 1 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:24453]
6	M/R	x	x
82	P/L	x	x
		MRS2	MRS2 magnesium homeostasis factor homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:13785]
257	D/N	x	x
		CLPTM1L (2 of 2)	CLPTM1-like [Source:HGNC Symbol;Acc:24308]
		PTK2	PTK2 protein tyrosine kinase 2 [Source:HGNC Symbol;Acc:9611]
		NDRG1 (2 of 2)	N-myc downstream regulated 1 [Source:HGNC Symbol;Acc:7679]
		PTP4A3	protein tyrosine phosphatase type IVA, member 3 [Source:HGNC Symbol;Acc:9636]
		ZFAT	zinc finger and AT hook domain containing [Source:HGNC Symbol;Acc:19899]
		MTBP (1 of 2)	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa [Source:HGNC Symbol;Acc:7417]
264	A/T	MTBP (2 of 2)	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa [Source:HGNC Symbol;Acc:7417]
276	P/S	MTBP (2 of 2)	
480	P/T	MTBP (2 of 2)	
		SCGN	secretagogin, EF-hand calcium binding protein [Source:HGNC Symbol;Acc:16941]
		LRRC16A (2 of 2)	leucine rich repeat containing 16A [Source:HGNC Symbol;Acc:21581]
		TNFRSF6B (2 of 2)	tumor necrosis factor receptor superfamily, member 6b, decoy [Source:HGNC Symbol;Acc:11921]
		TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b [Source:HGNC Symbol;Acc:11909]

Table 6 (continued 4)

Non-synonymous change in a.a.		gene	description
a.a. position	a.a. change		
163	G/R	COLEC10	collectin sub-family member 10 (C-type lectin) [Source:HGNC Symbol;Acc:2220]
134	K/R	MTSS1 (2 of 2)	metastasis suppressor 1 [Source:HGNC Symbol;Acc:20443]
266	S/I	MTSS1 (2 of 2)	
		TMEM65 (1 of 2)	transmembrane protein 65 [Source:HGNC Symbol;Acc:25203]
		MBOAT1 (2 of 2)	membrane bound O-acyltransferase domain containing 1 [Source:HGNC Symbol;Acc:21579]
		CDKAL1	CDK5 regulatory subunit associated protein 1-like 1 [Source:HGNC Symbol;Acc:21050]
		C3VV08_ORYLA	SRY-box containing gene 4 [Source:RefSeq peptide;Acc:NP_001158353]
		NRSN1	neurensin 1 [Source:HGNC Symbol;Acc:17881]
		DCDC2	doublecortin domain containing 2 [Source:HGNC Symbol;Acc:18141]
		SLC6A3	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3 [Source:HGNC Symbol;Acc:11049]
462	S/R	LPCAT1	lysophosphatidylcholine acyltransferase 1 [Source:HGNC Symbol;Acc:25718]
278	S/T	IRX4	iroquois homeobox 4 [Source:HGNC Symbol;Acc:6129]
		IRX2	iroquois homeobox 2 [Source:HGNC Symbol;Acc:14359]
		IRX1	iroquois homeobox 1 [Source:HGNC Symbol;Acc:14358]
8	Y/F	ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif, 16 [Source:HGNC Symbol;Acc:17108]
		KIAA0947	KIAA0947 [Source:HGNC Symbol;Acc:29154]
146	I/V	x	x
227	W/C	x	x
231	W/C	x	x
325	S/Y	x	x
		COBL	cordon-bleu homolog (mouse) [Source:HGNC Symbol;Acc:22199]
105	R/M	x	x
139	V/I	GRB10	growth factor receptor-bound protein 10 [Source:HGNC Symbol;Acc:4564]
		C0SQJ0_ORYLA	aromatic-L-amino-acid decarboxylase [Source:RefSeq peptide;Acc:NP_001153917]
		ENTPD3	ectonucleoside triphosphate diphosphohydrolase 3 [Source:HGNC Symbol;Acc:3365]
389	G/S	RBMS3 (2 of 2)	RNA binding motif, single stranded interacting protein 3 [Source:HGNC Symbol;Acc:13427]
362	G/S	RBMS3 (2 of 2)	

a.a, amino acid; x, predicted gene

Figure 13. Generation of a congenic line by speed congenic method

N2, HNI-II strain; R, Hd-rR-II1 strain.

Figure 14. Comparison of response stability index among congenic lines and the founder strains

N2, HNI-II; R, Hd-rR-II1. Bars represent SEM. **: $p<0.01$ by Scheffe's F test.

Figure 15. Pairwise QTL analysis for epistatic and additive effects for the response stability index

The horizontal and vertical axes of the panels indicate genetic positions. Above the diagonal represents analysis of the epistatic effects (comparison between full QTL model and additive QTL model) and below the diagonal represents analysis of the additive effects (comparison between additive QTL model and single QTL model). Color scales indicate LOD scores for QTL pairs; note that the scale is different for epistatic effects (left) and additive effects (right). Arrows at the sides of the scale indicate the thresholds (p=0.05). Magenta circle in the panel indicates the significant pair of additive QTLs, which was detected on chromosomes 16 and 21.

Figure 16. Close-up of additive QTL analysis result on chromosomes 15-24

Color scale indicates LOD score. Magenta circle indicates the significant pair of additive QTLs on chromosomes 16 and 21.

General discussion

This body of work is the first to identify QTLs for startle response properties and to demonstrate that a certain genomic region is sufficient to explain a part of the intraspecific behavioral difference without interactions with other regions. The effect of the identified QTL (26.7% variance explained) was fairly higher than that of behavioral QTL in previous rodent studies (mostly ≦10%, ~5% on average (Flint, 2003, Flint, Valdar, et al. 2005)). This was achieved using inbred strains of medaka with genetically diverse genetic backgrounds and establishing the behavioral quantification paradigm with high sensitivity and reproducibility for the behavioral properties.

The next important step is to identify the responsible genes or sequences within the candidate region. Although it has been difficult to identify specific responsible genes underlying behavioral QTLs due to weakness of effect of each QTL, complex epistasis (Flint, 2003) and clustering of QTLs with opposite effects (Kato, et al. 2014), I expect this will be feasible using the advanced molecular genetic techniques available for medaka, such as transgenic and genomic editing (Ansai, Inohaya, et al. 2014, Ansai and Kinoshita, 2014, Ansai, Sakuma, et al. 2013, Okuyama, Isoe, et al. 2013), which allow us to manipulate and dissect a certain genomic region precisely. Given that F1 between HNI-II and Hd-rR-II1 had a phenotype similar to that of Hd-rR-II1 (Figure 11C), an Hd-rR-II1 type allele of the responsible gene is thought to inhibit or suppress the HNI-II phenotype (low level of attenuation in response rate) expressed by an HNI-II type allele. Generation of Hd-rR-II1 with partial deletion in the candidate loci by the targeted genome editing method with CRISPR/Cas9 system (Ansai and Kinoshita, 2014) and behavioral testing of F1 between HNI-II and the mutant Hd-rR-II1 may enable us to identify the responsible gene.

From studies with goldfish and zebrafish suggest that thresholds to startling stimuli of the C-start response are regulated by the excitability of Mauthner cells and their homologs, pairs of

neurons that control the all-or-none startle response (Eaton, Lee and Foreman 2001, Medan and Preuss 2014, also see Figure 9). I expect that genes responsible for the response stability index, which would be identified in future genetic analyses, are involved in regulating the excitatory and inhibitory balance of those neurons or inputs from optic neurons to those central neurons. In mammals and other teleost species, an attenuated startle response (in probability and/or amplitude) to repeated stimuli is recoverable by a different stimulus (Rankin, et al. 2009). Because this feature of startle responses cannot be explained by desensitization and sensitization of sensory or motor neurons, it is unlikely that the responsible genes regulate excitation of optic neurons or outputs from the central neurons, although I cannot exclude possibility of this at the present time. Transportation of eyes between the inbred lines could help us to examine whether retinal properties have some influences on the behavioral differences. If sensitivity or adaptivity of retinal neurons were responsible for the "timidness", it would present a concept that ways to percept circumstances have influences on ways to deal with the circumstances.

This work is also the first to identify QTLs contributing to behavioral differences among medaka inbred strains. The originality of this work is in its focus on behavioral diversity or "animal personality" in medaka. The various inbred strains generated from the same and different populations exhibited significantly different startle response properties, which might be related to their behavioral tendencies (Figure 17A). Behavioral characterization of the congenic lines and their background strains across different contexts (e.g. foraging, mating, schooling etc.) may support the hypothesis.

Among the inbred strains used in this study, HNI-II and HNI-I showed a detectable difference in the response stability index. Considering that they are generated from different pairs in the same local-wild population, this inter-strain behavioral difference might have originated from behavioral and genetic differences among individuals within a wild population. Identification of the

responsible genes will allow us to investigate the distribution of genetic polymorphisms within and among populations living in the wild.

Throughout these doctoral studies, I become very interested in the influence of inherent personalities of group members on collective behaviors. For example, given the previous finding that fast individuals in a medaka group could induce alignment and coordinated swimming of group members (Ochiai, et al. 2013), I assumed that a mixed group of individuals with high responsiveness (like HNI-II) and low responsiveness (like Hd-rR-II1) would form a leader-follower relationship in coordinated escape and more successfully avoid danger than a homogeneous group of individuals with low responsiveness (Figure 17B). Such coexistence could be profitable not only for individuals with low responsiveness, but also for ones with high responsiveness, if such "timid" individuals were in turn led by "bold" individuals in other behaviors such as foraging (Figure 17B). When foraging together, "timid" individuals may follow "bold" individuals that go actively out of a hiding place as suggested in the stickleback study (Harcourt, et al. 2009), and might become more successful in finding food than when foraging only with "timid" individuals. These behavioral tests can be developed using inbred medaka strains and the established congenic lines, providing insight into the maintenance mechanism of behavioral diversity within a population. Moreover, identification of the genes responsible for the startle response properties might allow us to track the process of selection and maintenance of genetic polymorphisms within a population under controlled laboratory environments.

This study revealed the potential of medaka as a new model animal for behavioral genetics as well as evolutionary behavioral ecology. I hope that this work will contribute to clarification of molecular and neural background of "animal personalities" and enhance our understanding of the biologic significance of behavioral diversity within animal societies.

Figure 17. Assumed models and future perspectives

(A) Relationship between startle/escape properties and dimensions of “animal personalities”. (B) Interactions between “timid” (left) and “bold” (right) individuals in a group in escaping and foraging.

Ethics statement

The work in this thesis was conducted using protocols approved by the Animal Care and Use Committee of the University of Tokyo (permit number: 12–07). All surgery was performed under cold anesthesia, and all efforts were made to minimize suffering.

References

Ahsan B, Kobayashi D, Yamada T, Kasahara M, Sasaki S, Saito TL, Nagayasu Y, Doi K, Nakatani Y, Qu W, Jindo T, Shimada A, Naruse K, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Kohara Y, Morishita S (2008) UTGB/medaka: genomic resource database for medaka biology. Nucleic Acids Research 36: D747-752.

Ansai S, Kinoshita M (2014) Targeted mutagenesis using CRISPR/Cas system in medaka. Biology Open 3: 362-371.

Ansai S, Inohaya K, Yoshiura Y, Schartl M, Uemura N, Takahashi R, Kinoshita M (2014) Design, evaluation, and screening methods for efficient targeted mutagenesis with transcription activator-like effector nucleases in medaka. Development Growth and Differentiation 56: 98-107.

Ansai S, Sakuma T, Yamamoto T, Ariga H, Uemura N, Takahashi R, Kinoshita M (2013) Efficient targeted mutagenesis in medaka using custom-designed transcription activator-like effector nucleases. Genetics 193: 739-749.

Bell AM (2005) Behavioural differences between individuals and two populations of stickleback (*Gasterosteus aculeatus*). Journal of Evolutionary Biology 18: 464–473.

Bergmuller R, Taborsky M (2010) Animal personality due to social niche specialisation. Trends in Ecology and Evolution 25: 504-511.

Bouchard TJ, Jr., Loehlin JC (2001) Genes, evolution, and personality. Behavior Genetics 31: 243-273.

Broman KW, Wu H, Sen Ś, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19: 889-890.

Carlborg Ö & Haley SC (2004) Epistasis: too often neglected in complex trait studies? Nature Reviews Genetics 5, 618-625.

Carter AJ, Feeney WE, Marshall HH, Cowlishaw G, Heinsohn R (2013) Animal personality: what are behavioural ecologists measuring? Biological Review 88: 465-475.

Coleman K, Wilson DS (1998) Shyness and boldness in pumpkinseed sunfish: individual differences are context-specific. Animal Behaviour, 56: 927–936.

Eaton RC, editor (1984) Neural Mechanisms of Startle Behavior: Springer US. 377 p.

Eaton R, Lavender W, Wieland C (1981) Identification of Mauthner-initiated response patterns in goldfish: Evidence from simultaneous cinematography and electrophysiology. Journal of Comparative Physiology 144: 521-531.

Eaton RC, Lee RKK, Foreman MB (2001) The Mauthner cell and other identified neurons of the brainstem escape network of fish. Progress in Neurobiology 63: 467–485

Fidler AE, van Oers K, Drent PJ, Kuhn S, Mueller JC, Kempenaers B (2007) Drd4 gene polymorphisms are associated with personality variation in a passerine bird. Proceedings of the Royal Society B: Biological Sciences 274: 1685-1691.

Flint J (2003) Analysis of quantitative trait loci that influence animal behavior. Journal of Neurobiology 54: 46-77.

Flint J, Valdar W, Shifman S, Mott R (2005) Strategies for mapping and cloning quantitative trait genes in rodents. Nature Reviews Genetics 6: 271-286.

Glowa J, Hansen C (1994) Differences in response to an acoustic startle stimulus among forty-six rat strains. Behavior Genetics 24: 79-84.

Godin GJ (1997) Evading predators: In: Godin GJ, editor. Behavioural ecology of teleost fishes. , Oxford, UK: Oxford University Press. pp. 191–236.

Gosling SD (2001) From mice to men: what can we learn about personality from animal research? Psychological Bulletin 127: 45-86.

Grinsted L, Pruitt JN, Settepani V, Bilde T (2013). Individual personalities shape task differentiation in a social spider. Proceedings of the Royal Society B: Biological Sciences 280: 20131407.

Gwilliam GF (1963) The mechanism of the shadow reflex in Cirripedia. I. Electrical activity in the supraesophageal ganglion and ocellar nerve. Biological Bulletin 125: 470-485.

Harcourt JL, Ang TZ, Sweetman G, Johnstone RA, Manica A (2009) Social feedback and the emergence of leaders and followers. Current Biology 19: 248-252

Herbert-Read JE, Krause S, Morrell LJ, Schaerf TM, Krause J, Ward AJW (2013) The role of individuality in collective group movement. Proceedings of the Royal Society of London B: Biological Sciences 280: 20122564.

Hirayama M, Mukai T, Miya M, Murata Y, Sekiya Y, Yamashita T, Nishida M, Watabe S, Oda S, Mitani H (2010) Intraspecific variation in the mitochondrial genome among local populations of Medaka Oryzias latipes. Gene 457: 13-24.

Hyodo-Taguchi Y (1983) Effects of UV irradiation on embryonic development of different inbred strains of the fish *Oryzias latipes*. Journal of Radiation Research 24: 221-228.

Hyodo-Taguchi Y (1990) Inbred strains of the medaka and their characters.(In Japanese); Egami N, Yamagami K, Shima A, editors. Tokyo, Japan: Tokyo University Press. pp. 129–142.

Hyodo-Taguchi Y(1996) Inbred strains of the medaka, *Oryzias talipes*: The Fish Biology Jounal MEDAKA 8: 11-14.

Imada H, Hoki M, Suehiro Y, Okuyama T, Kurabayashi D, Shimada A, Naruse K, Takeda H, Kubo T, Takeuchi H (2010) Coordinated and cohesive movement of two small conspecific fish induced by eliciting a simultaneous optomotor response. PLoS One 5: e11248.

Ishikawa Y, Yoshimoto M, Yamamoto N, Ito H (1999) Different brain morphologies from different genotypes in a single teleost species, the medaka (*Oryzias latipes*). Brain Behavior and Evolution 53: 2-9.

Kasahara M, Naruse K, Sasaki S, Nakatani Y, Qu W, Ahsan B, Yamada T, Nagayasu Y, Doi K, Kasai Y, Jindo T, Kobayashi D, Shimada A, Toyoda A, Kuroki Y, Fujiyama A, Sasaki T, Shimizu A, Asakawa S, Shimizu N, Hashimoto S, Yang J, Lee Y, Matsushima K, Sugano S, Sakaizumi M, Narita T, Ohishi K, Haga S, Ohta F, Nomoto H, Nogata K, Morishita T, Endo T, Shin-I T, Takeda H, Morishita S, Kohara Y (2007) The medaka draft genome and insights into vertebrate genome evolution. Nature 447: 714-719.

Katsumura T, Oda S, Mano S, Suguro N, Watanabe K, Mitani H, Oota H, Kawamura S (2009) Genetic differentiation among local populations of medaka fish (*Oryzias latipes*) evaluated through grid- and deme-based sampling. Gene 443: 170-177.

Kimura T, Naruse K (2010) M-marker 2009, a marker set for mapping medaka mutants using PCR length polymorphisms with an automated microchip gel electrophoresis system. Biotechniques 49: 582-583.

Kimura T, Shimada A, Sakai N, Mitani H, Naruse K, Takeda H, Inoko H, Tamiya G, Shinya M (2007) Genetic analysis of craniofacial traits in the medaka. Genetics 177: 2379-2388.

Kimura T, Shinya M, Naruse K (2012) Genetic analysis of vertebral regionalization and number in medaka (*Oryzias latipes*) inbred lines. G3 (Bethesda) 2: 1317-1323.

Kimura T, Yoshida K, Shimada A, Jindo T, Sakaizumi M, Mitani M, Naruse K, Takeda H, Inoko H, Tamiya G, Shinya M (2005) Genetic linkage map of medaka with polymerase chain reaction length polymorphisms. Gene 363: 24-31.

Kato S, Ishii A, Nishi A, Kuriki S, Koide T (2014) Segregation of a QTL cluster for home-cage activity using a new mapping method based on regression analysis of congenic mouse strains. Heredity 113: 416-423

Kobayashi D, Takeda H (2008) Medaka genome project. Briefings in Functional Genomics and Proteomics 7: 415-426.

Krause J, Ruxton GD (2002) Living in Groups. Oxford, UK: Oxford University Press.

Lucas MD, Drew RE, Wheeler PA, Verrell PA, Thorgaard GH (2004) Behavioral Differences Among Rainbow Trout Clonal Lines. Behavior Genetics 34: 355-365

Mackay TF, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. Nature Reviews Genetics 10: 565-577.

Matsuda M, Shinomiya A, Kinoshita M, Suzuki A, Kobayashi T, Paul-Prasanth B, Lau EL, Hamaguchi S, Sakaizumi M, Nagahama Y (2007) The *DMY* gene induces male development in genetically female (XX) medaka fish. Proceedings of Natural Academy of Sciences of the United States of America 104: 3865-3870

Matsumoto Y, Fukamachi S, Mitani H, Kawamura S (2006) Functional characterization of visual opsin repertoire in Medaka (*Oryzias latipes*). Gene 371: 268–278.

Matsumoto Y, Oota H, Asaoka Y, Nishina H, Watanabe K, Bujnicki JM, Oda S, Kawamura S, Mitani H (2009) Medaka: a promising model animal for comparative population genomics. BMC Research Notes 2: 88.

Medan V, Preuss T (2014) The Mauthner-cell circuit of fish as a model system for startle plasticity. Journal of Physiology-Paris 108: 129-140.

Michelena P, Jeanson R, Deneubourg J-L, Sibbald AM (2010) Personality and collective decision-making in foraging herbivores. Proceedings of the Royal Society of London B: Biological Sciences 277: 1093-1099.

Moriwaki K, Miyashita N, Mita A, Gotoh H, Tsuchiya K, Kato H, Mekada K, Noro C, Oota S, Yoshiki A, Obata Y, Yonekawa H, Shiroishi T (2009) Unique inbred strain MSM/Ms established from the Japanese wild mouse. Experimental Animals 58: 123-134.

Müller L, Grath S, von Heckel K, Parsch J (2012) Inter- and Intraspecific Variation in Drosophila Genes with Sex-Biased Expression. International Journal of Evolutionary Biology 2012: Article ID 963976.

Naruse K, Fukamachi S, Mitani H, Kondo M, Matsuoka T, Hanamura N, Morita Y, Hasegawa K, Nishigaki R, Shimada A, Wada H, Kusakabe T, Suzuki N, Kinoshita M, Kanamori A, Terado T, Kimura H, Nonaka M, Shima A (2000) A detailed linkage map of medaka, *Oryzias latipes*: comparative genomics and genome evolution. Genetics 154: 1773-1784.

Ochiai T, Suehiro Y, Nishinari K, Kubo T, Takeuchi H (2013) A new data-mining method to search for behavioral properties that induce alignment and their involvement in social learning in medaka fish (*Oryzias latipes*). PLoS One 8: e71685.

van Oers K, Drent PJ, de Goede P, van Noordwijk AJ (2004) Realized heritability and repeatability of risk-taking behaviour in relation to avian personalities. Proceedings of the Royal Society of London. Series B 271: 65-73.

Okuyama T, Isoe Y, Hoki M, Suehiro Y, Yamagishi G, Naruse K, Kinoshita M, Kamei Y, Shimizu A, Kubo T, Takeuchi H (2013) Controlled Cre/loxP site-specific recombination in the developing brain in medaka fish, *Oryzias latipes*. PLoS One 8: e66597.

Okuyama T, Yokoi S, Abe H, Isoe Y, Suehiro Y, Imada H, Tanaka M, Kawasaki T, Yuba S, Taniguchi Y, Kamei Y, Okubo K, Shimada A, Naruse K, Takeda H, Oka Y, Kubo T, Takeuchi H (2014) A neural mechanism underlying mating preferences for familiar individuals in medaka fish. Science 343: 91-94.

Perez-Escudero A, Vicente-Page J, Hinz RC, Arganda S, de Polavieja GG (2014) idTracker: tracking individuals in a group by automatic identification of unmarked animals. Nature Methods 11: 743-748.

Quinn JL, Cresswell W (2005) Escape response delays in wintering redshank, *Tringa tetanus* flocks: perceptual limits and economic decisions. Animal Behavior 69; 1285–1292.

Rankin CH, Abrams T, Barry RJ, Bhatnagar S, Clayton D, Colombo J, Coppola G, Geyer MA, Glanzman DL, Marsland S, McSweeney F, Wilson DA, Wu C, Thompson RF (2009). Habituation Revisited: An Updated and Revised Description of the Behavioral Characteristics of Habituation. Neurobiology of Learning and Memory 92: 135–138.

Réale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ (2007) Integrating animal temperament within ecology and evolution. Biological Review 82: 291-318.

Russell EM (1967) Changes in the behaviour of Lebistes reticulatus upon a repeated shadow stimulus. Animal Behavior 15: 574-585.

Ruxton GD, Sherratt TN, Speed MP (2004) Avoiding attack: the evolutionary ecology of crypsis, warning signals, and mimicry. Oxford, UK: Oxford University Press.

Sasado T, Tanaka M, Kobayashi K, Sato T, Sakaizumi M, Naruse K (2010) The National BioResource Project Medaka (NBRP Medaka): an integrated bioresource for biological and biomedical sciences. Experimental Animals 59: 13-23.

Sato T, Endo T, Yamahira K, Hamaguchi S, Sakaizumi M (2005) Induction of Female-to-Male Sex Reversal by High Temperature Treatment in Medaka, *Oryzias latipes*. Zoological Science 22: 985-988.

Sih A, Bell A, Johnson JC (2004) Behavioral syndromes: an ecological and evolutionary overview. Trends in Ecology and Evolution 19: 372-378.

Smith BR, Blumstein DT (2008) Fitness consequences of personality: a meta-analysis. Behavioral Ecology 19: 448-455.

Spivakov M, Auer TO, Peravali R, Dunham I, Dolle D, Fujiyama A, Toyoda A, Aizu T, Minakuchi Y, Loosli F, Naruse K, Birney E, Wittbrodt J (2014) Genomic and phenotypic characterization of a wild medaka population: towards the establishment of an isogenic population genetic resource in fish. G3 (Bethesda) 4: 433-445.

Takahashi A, Shiroishi T, Koide T (2014) Genetic mapping of escalated aggression in wild-derived mouse strain MSM/Ms: association with serotonin-related genes. Frontiers in Neuroscience 8:156.

Takeda H (2008) Draft genome of the medaka fish: a comprehensive resource for medaka developmental genetics and vertebrate evolutionary biology. Development Growth and Differentiation 50 Supplement s1: S157-166.

Togawa K, Moritani M, Yaguchi H, Itakura M (2006) Multidimensional genome scans identify the combinations of genetic loci linked to diabetes-related phenotypes in mice. Human Molecular Genetics 15: 113-128.

Tsuboko S, Kimura T, Shinya M, Suehiro Y, Okuyama T, Shimada A, Takeda H, Naruse K, Kubo T, Takeuchi H (2014) Genetic Control of Startle Behavior in Medaka Fish. PLoS One 9: e112527.

Wade CM, Kulbokas EJ 3rd, Kirby AW, Zody MC, Mullikin JC, Lander ES, Lindblad-Toh K, Daly MJ (2002) The mosaic structure of variation in the laboratory mouse genome. Nature 420: 574-578.

Ward AJW, Thomas P, Hart PJB, Krause J (2004) Correlates of boldness in three-spined sticklebacks (*Gasterosteus aculeatus*). Behavioral Ecology and Sociobiology 55: 561-568.

Webster MM, Ward AJ (2011) Personality and social context. Biological Reviews of Camblidge Philosophical Society 86: 759-773.

Wilson DS, Clark AB, Coleman K, Dearstyne T (1994) Shyness and boldness in humans and other animals. Trends in Ecology and Evolution 9: 442-446.

Wong GT, (2002) Speed congenis: applications for transgenic and knock-out mouse strains. Neuropeptides 36: 230-236.

Acknowledgments

First and foremost, I would like to express my greatest gratitude to Dr. Takeo Kubo (Professor, at the University of Tokyo), for cultivating my research sense and attitude toward science. I also would like to extend my appreciation to Dr. Hideaki Takeuchi (Assistant Professor, at the University of Tokyo) for providing me the sustained impetus to continue to progress in my research.

I greatly appreciate Dr. Kiyoshi Naruse (Associate Professor at National Institute for Basic Biology: NIBB) and Dr. Minori Shinya at Keio University (formerly National Institute of Genetics) for their continuous support and guidance. I am also thankful for the informative discussions with Dr. Tetsuaki Kimura at the NIBB.

I also thank the National BioResource Project Medaka, which is supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for supplying the medaka inbred strains. My studies were also supported by the NIBB Priority Collaborative Research Project (10-104), a Grant-in-Aid for Scientific Research on Innovative Areas (Comprehensive Brain Science Network), and the Graduate Program for Leaders in Life Innovation (GPLLI), The University of Tokyo Life Innovation Leading Graduate School, all of which are from MEXT; and a Grant-in-Aid from the Japan Society for Promotion of Science (JSPS) fellowship.

I appreciate Dr. Yusuke Takehana and Dr. Takao Sasado at NIBB for their technical assistance. I am also grateful for the devoted assistance of Ms. Hisako Kohno at the University of Tokyo, and Ms. Yukari Koike and all the staff in the Laboratory of BioResorces at NIBB in fish maintenance.

I am very thankful for the inspiring discussions and the laboratory camaraderie with my former and present colleagues in the Laboratory of Physiological Chemistry at the University of Tokyo.

Lastly, I would like to express my heartfelt thanks to my family, both by blood and by marriage, for raising me, growing together, and respecting my individual personality.