学位論文

Genetic studies on the role of vasotocin and isotocin in social motivation of medaka fish

（メダカの社会性動機付けにおける

バソトシンとイソトシンの機能に関する遺伝学的研究）

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Abbreviations

VT : vasotocin

VP : vasopressin

dpf : day post fertilization

GFP : Green Fluorescent Protein

HRM : High Resolution Melting curve

IT: isotocin

ITR1 : isotocin receptor 1

KO: knockout

OMR : Optomotor Response

OT : oxytocin

PGC : Primordial Germ Cell

RFP: Red Fluorescent Protein

Tg : transgenic line

TILLING : Targeting Induced Local Lesion IN Genomes

TALEN : Transcription Activator-Like Effector Nucleases

V1a1 : V1a type vasotocin receptor 1

V1a2 : V1a type vasotocin receptor 2

Abstract

In various social animals, including humans, social members modify their behaviors toward other group members based on their social context. An extensive literature has focused on the neural/molecular mechanisms underlying the innate and simple social behaviors, such as courtship behavior and aggression, in dyadic interactions using various animal species. The genetic/molecular mechanisms underlying more complex social behaviors, such as triadic relationships and social memory, however, remain largely unknown. One reason for the difficulty of identifying them is that the model organisms that are commonly used for molecular genetics, such as fruit fly and zebrafish, do not exhibit such prominently complex social behaviors.

To circumvent this issue, I have focused on medaka fish, which exhibit various social behaviors. In my master course study, I found that when one medaka female and two medaka males are put together in a tank, one male tends to keep its position between the female and the other male and prevents the other male from approaching the female. I hypothesized that this is a mate-guarding behavior (i.e., preventing other males from mating with their potential or former mates by maintaining close proximity) exhibited by male medaka in the triad, and demonstrated it by establishing a new behavioral assay system to quantify this behavior and accessing the adaptive fitness of the dominant males in this behavior. This was the first demonstration that a model animal commonly used for molecular genetics exhibits mate-guarding behavior in a triad, which allows us to analyze the underlying neural/molecular mechanisms using the molecular genetic methods.

In my doctoral course study, I intended to investigate the molecular/neural

mechanisms underlying the mate-guarding behavior of the male medaka. First, I examined whether one of neuropeptides, vasotocin (VT) was involved in mate-guarding of male medaka. VT and vasopressin (VP), a mammalian homolog of VT are known to regulate male typical social behaviors, such as aggressive behavior and pair-bonding (Donaldson et al., 2008). Pharmacologic studies of other types of fish have implicated the involvement of the VT system in courtship behaviors and aggressive behaviors (Semsar et al., 2001). I generated medaka mutants with alterations of VT and its receptors (V1a1, V1a2) genes and showed that *vt* and *V1a2* are required for normal mate-guarding behavior. In addition, a behavioral analysis of courtship behaviors in a dyad relationship and aggressive behaviors within an all-male group showed that *vt* mutant males exhibit decreased sexual motivation but normal aggression. In contrast, heterozygote *V1a2* mutant males displayed decreased aggression, but normal mate-guarding and courtship behavior. These findings suggested that impaired mate-guarding motivations in *vt* and *V1a2* homozygote mutants are due to the loss of sexual behaviors toward females rather than the loss of the competitive behaviors toward rival males. Furthermore, the different behavioral phenotypes among *vt, V1a2* heterozygote and *V1a2* homozygote mutants suggest that the redundant systems activate V1a2, and the endogenous ligands activating the receptor may differ according to the social context.

I also generated medaka mutants for isotocin (IT) and its receptor (ITR1) genes, and demonstrated that these homozygote mutant males tend to be dominant in mate-guarding. Thus, the IT system may suppress mate-guarding behavior. In addition, these mutant males exhibit normal courtship and aggressive behaviors, suggesting that the IT system is involved in the molecular mechanism underlying mate-guarding in a triadic relationship.

Furthermore, I found that social familiarity has strong effects on various social

behaviors (e.g., aggressive behavior, courtship behavior, and mate-guarding behavior) in *it* mutants, but not those in wild-type males. *it* mutant males exhibit such social behaviors toward unfamiliar individuals less frequently than toward familiar individuals. Furthermore, defects of social interactions toward unfamiliar individuals in *it* mutant males was recovered by “social familiarization”, in which the fish were allowed to live together in the same tank for about 3 weeks. Thus, *it* mutant males can discriminate between unfamiliar and familiar individuals, and their social motivation is based on familiarity. Based on these findings, I propose new functions of the VT and IT systems in highly social behaviors, which might be conserved among vertebrates.

General Introduction

Various social animals, including humans, select behaviors based on their social context/interactions. For example, males exhibit courtship behavior toward their potential sexual mates (intersexual interaction) and aggressive behavior toward rival males (intrasexual interaction) (Anderson et al., 2014). In addition, some animals can recognize other conspecific individuals (social recognition) within the group and change their social behavior toward other members according to the social familiarity (Ferguson et al., 2000) or hierarchy (Hofmann et al., 1999). This behavioral motivation based on the individual recognition and social context is essential for the social animals to more efficiently adapt to their social lives.

An extensive literature has focused on the neural/molecular mechanisms underlying these dyadic social interactions, such as intersexual interaction (courtship behavior) and intrasexual competition (aggressive behavior). In many animals, the involvements of vasopressin (VP) and oxytocin (OT) homologs in these behaviors have been extensively investigated (Donaldson et al., 2008; Winslow et al., 2002). The neural/molecular mechanisms underlying more complex behavior, such as triadic behavior and social motivation according to the social familiarity, however, are largely unknown.

Among such triadic behavior, the mate-guarding behavior, which is defined as "activity that prevents other males from mating with their potential or former mates by maintaining close proximity", has been studied exclusively in behavioral ecology and phylogeny (Beecher et al., 1979; Tutin, 1979). However, the underlying neural/molecular mechanisms have remained largely unknown because of the lack of behavioral systems that robustly elicit this type of complex behavior under laboratory conditions for any genetically

manipulatable model organisms.

To explore this issue, I focused on medaka fish (*Oryzias latipes*). Medaka are small oviparous freshwater teleosts native to Japan, South Korea, and China. The use of medaka fish for the analysis of social behavior and genetic dissection of neural circuits has many advantages, including: 1) easy breeding with a relatively short generation time (2 ~ 3 months); 2) availability of advanced genetic/molecular techniques (Furutani-Seiki et al., 2004; Taniguchi et al., 2006; Naruse et al., 2004; Ansai et al., 2013); 3) short reproductive cycle (24 h) with adult medaka exhibiting mating behaviors every morning (Kobayashi et al., 2012); and 4) various types of observable highly social behaviors (Okuyama et al., 2014; Imada et al., 2010).

In my master course study, I found that medaka males rubustly and prominently exhibit mate-guarding behavior in a triadic relationship of two males and one female and established a novel assay system to quantify this behavior (Chapter 1). Using this behavioral asssay system, I first investigated the genetic/molecular mechanisms underlying the mate-guarding behavior in medaka (Chapters 2 & 3). I assumed that the motivation for mate-guarding consisted of sexual motivation toward females (intersexual motivation) and competitive motivation towards other males (intrasexual motivation), and these two motivation cooperatively induced mate-guarding. Therefore, as candidate molecular systems that are involved in mate-guarding behavior, I focused on vasotocin (VT), a non-mammalian homolog of vasopressin, and isotocin (IT), a teleost homolog of oxytocin, because these neuropeptides are known to be involved in the courtship behavior (intersexual interaction) and aggressive behavior (intrasexual interaction) in many animals (Donaldson 2008). The involvement of VT and IT in social behaviors has been implicated based on pharmacologic

methods (Semsar et al., 2001; Reddon et al., 2012), but it has not been studied at the genetic level in any non-mammalian species. Therefore, I intended to use molecular genetic tools, including the TILLING (Targeting Induced Local Lesions IN Genomes) (Taniguchi et al., 2006) and TALEN (Transcription Activator-Like Effector Nucleases) methods (Ansai et al., 2013), to genetically examine how the VT and IT pathways are involved in mate-guarding behaviors (Chapters 2 and 3).

In mammals, OT knockout (KO) male mice cannot change their behavior according to social familiarity (Young WS et al., 2003). Therefore, I checked whether IT-related gene mutant medaka males exhibited abnormal social motivation according to social familiarity in mate-guarding, courtship and aggressive behaviors (Chapter 4). To my knowledge, a series of my experiments in this thesis is the first genetic study demonstrating the molecular mechanisms underlying mate-guarding behavior in triadic relationships, as well as the possible key involvement of IT in social motivation based on social familiarity.

Chapter 1

Establishment of a novel behavioral assay system to quantify mate-guarding behavior in medaka

Introduction

Mating strategies have evolved to increase inclusive fitness via sexual selection. In many animals, females choose their mating partners based on some secondary characteristics (intersexual selection) and males compete with each other for mating (intrasexual selection). Although most attention has been paid to either intersexual or intrasexual selection (Anderson et al., 2014), some studies have focused on mate-guarding behaviors, which include both intersexual and intrasexual selection (Sherman et al., 1989).

Mate-guarding behavior is defined as "activity that prevents other males from mating with their potential or former mates by maintaining close proximity". For example, in some monogamous avian species, the males follow the females and beat off other rival males attempting to approach the female (Birkhead et al., 1982). In insects, such as the dragonfly, the males continue to hang on the females after copulation (Hasegawa et al., 2000) to prevent rivals from copulating with the female. Mate-guarding behavior is also reported in various primates (Hasegawa et al., 2000). Lack of attention to either a mating partner or rival males in mate-guarding would allow the rivals to approach and mate with the partner, known as sneaking (Zamudio et al., 2000) and extra-pair copulation (Birkhead et al., 1988; van Dongen, 2008; Ewen et al., 1993). In fact, ecologic studies indicate that mate-guarding is required to increase individual male fitness in some vertebrates (Sherman, 1989). Although mate-guarding behavior has been studied exclusively in behavioral ecology and phylogeny, the neural/molecular mechanism has remained largely unknown. To study this issue, I focused on medaka fish, a model animal for molecular genetics, and found that medaka males exhibit mate-guarding behavior. In this chapter, I describe how I established a novel behavioral test to

assess mate-guarding in medaka. It is the first report that a model animal exhibits robust mate-guarding behavior under laboratory conditions and establishes an assay system that allows for investigation of the molecular/neural basis underlying mate-guarding.

Materials & Methods

Fish and breeding conditions

Medaka fish (*Oryzias latipes*, drR strain, nearly inbred strain) were maintained in like groups in plastic aquariums (13 cm x 19 cm x 12 cm (height)). All fish were hatched and bred in our laboratory. Sexually mature male and female adult medaka (more than 3 months after hatching), which showed the normal mating behavior and same body size as far as I observed, were used for the mating behavior assay. The water temperature was ~28°C and light was provided by ordinary fluorescent lamps for 14 hours per day (08:00 to 22:00).

Mate-guarding behavior assay

A detailed procedure is provided in Figure 2. One female and two males were placed in an aquarium (water depth was about 3-4cm, the light intensity was about 400-500 lx), and their behavior was recorded from the bottom of the aquarium , in the morning (10:00 to 12:00) and in the evening (20:00 to 21:00). Light was provided for 14 h per day (08:00 to 22:00). As a negative control group (merged group), I performed the same experiment using virtually merged trios, recording one female and two males, each placed in a separate aquarium (“Merge”). I converted video files into 21 image sequences per 5 s, and manually spotted the head and tail positions of the three medaka fish using ImageJ (NIH, Bethesda, MD, USA) to calculate the center positions as the body positions. The male whose mean distance from the female was shorter than that of the other male was “the near male” and the other was “the far male”. Based on the positions of the female (x_F, y_F), the far male (x_{Mf}, y_{Mf}), and the near male (x_{Mn}, y_{Mn}), the relative positions of the near male (X, Y) were calculated by the following

formula when the female and far male positions were defined as (0, 0) and (1, 0), respectively (See Figure 2).

$$X = \frac{\{(x_{Mf} - x_F)(x_{Mn} - x_F) + (y_{Mf} - y_F)(y_{Mn} - y_F)\}\sqrt{(x_{Mn} - x_F)^2 + (y_{Mn} - y_F)^2}}{\{(x_{Mf} - x_F)^2 + (y_{Mf} - y_F)^2\}^{\frac{3}{2}}}$$

$$Y = \frac{\{(x_{Mf} - x_F)(y_{Mn} - y_F) - (y_{Mf} - y_F)(x_{Mn} - x_F)\}\sqrt{(x_{Mn} - x_F)^2 + (y_{Mn} - y_F)^2}}{\{(x_{Mf} - x_F)^2 + (y_{Mf} - y_F)^2\}^{\frac{3}{2}}}$$

I spotted the relative positions of the near male and defined a circle with center (1/2, 0) and radius 1/2 as the "guarding circle". When the near male was within the guarding circle, the angle between the vectors from the near male to the female and from the near male to the far male was obtuse. The probability of being in the guarding circle was defined as the "guarding index" (See Figure 2).

Dominance test

A detailed procedure is provided in Figures 8. I used one *genotype A* male and one *genotype B* male and evaluated their mate-guarding behavior in the presence of a female. I measured the relative locations of the three fish and calculated the probability of the *genotype A* male being in the guarding circle when the female and *genotype B* male positions were defined as (0, 0) and (1, 0), respectively (Left). I defined this probability as the "guarding index of *genotype A*". In contrast, I also calculated the probability of the *genotype B* male being in the guarding circle when the female and *genotype A* male positions were defined as (0, 0) and (1, 0), respectively (Right). I defined this probability as the "guarding index of *genotype B*" and compared with that of *genotype A*. A higher guarding index indicates higher dominance in the

mate-guarding behavior compared with the other male (Figure 6).

Paternity and dominance tests

The paternity test was performed as described previously (Okuyama et al., 2014). One female and two males, one of which was the drR wild-type strain and the other of which was transgenic (Tg; *homozygote olvas*:*gfp*), were used for this test. To determine which male was dominant, I performed a dominance test over 6 days using same three fish (6 trials). The male whose mean guarding index for the 6 trials was significantly higher was considered dominant and the other was considered subordinate (Figure 8). Tg(*olvas*:*gfp*) were distinguished from drR by the GFP fluorescence of primordial germ cells, visible at 3 days post-fertilization, and even in the ventral area of the adult, by fluorescent microscopy (MZ16F, Leica, Tokyo, Japan). Therefore, I genotyped the progeny based on GFP detection.

Eye removal

Eye removal was performed as described previously. Anesthesia was achieved by exposure to MS222 (0.02%, Sigma-Aldrich, Tokyo). The eyes of the medaka (bilateral or unilateral) were surgically excised at the adult stage after cutting off the optic nerves. In the sham-operated medaka negative controls, only the lateral area of the eyes were slightly cut with a surgical knife. The fish were kept in water containing 0.7% NaCl for three days after surgery before being subjected to the mating behavior assay.

Results

Emergence of mate-guarding behavior in a triad relationship of medaka fish

In order to analyze the molecular/neural basis underlying mate-guarding behavior, it is essential to establish the behavioral assay system for quantifying mate-guarding behavior to detect the abnormal behavioral phenotypes of mutants. Therefore, I quantitatively analyzed mate-guarding behavior by calculating the relative position of three fish in a behavioral assay. When two males and one female were allowed to swim together in the morning (Figure 1A), the male-male competition led to the tendency of one male to maintain its position near the female and prevent the other male from approaching the female. I defined this behavior of the nearest male as "mate-guarding behavior" (Figure 1B). To quantify the degree of mate-guarding, I generated a novel index to represent the degree of mate-guarding of the focal fish (Figure 2). First, I measured time-series coordinate data of the three individual medaka fish (2 males and 1 female) for 100 s and calculated the relative locations of the three fish. The relative positions of the focal male were calculated when the fixed positions of the female and the other rival male were defined as (0, 0) and (1, 0), respectively. I spotted the relative positions of the focal male during the 100 s (once every 5 s; Figure 2). I then calculated the probability of the focal male being within the "guarding circle", defined as a circle with center (1/2, 0) and radius 1/2 (Figure 2). The presence of the focal male in the guarding circle indicated that the focal male occupied a dominant position, allowing him to both remain near the female and interfere with the rival (the other male). Thus, the probability of being within the guarding circle was considered to represent the degree of mate-guarding of the focal fish. Hereafter, I defined this probability as the "guarding index".

To evaluate whether mate-guarding emerges in a triangle relationship, I examined whether the "guarding index" of the nearest of the two males significantly increases based on the interactions of the three fish (guarding test, Figure 3A). The "near male" was defined as the male whose mean distance from the female during the 100-s recording period was shorter than that of the other male. The "far male" was defined as the other male. I then generated a "merged group" as a negative control, in which the three fish freely swam in individual aquaria without any social interaction. I separated three fish into three tanks of the same size, recorded the independent movement of each fish, and superimposed the data, which were used to calculate the virtual guarding index of the near male as a negative control. The guarding test using a wild-type medaka strain (drR) revealed a guarding index of 62.6% ± 4.6% for the near male, which was significantly higher than that of the merged control (30.4% ± 4.7%; Figures 3B: before, merge). We also performed guarding test using different size (small, large) and shape (rectangular vs round) tanks (Figure 3C). The guarding indices of the near male in the merged groups using different types of the tank, are almost same with that using normal rectangular tank and the guarding indices of the near male in experimental groups were significantly higher than those in merged groups in this test. This result suggested that mate-guarding robustly emerged irrespective of the geometric constraints of the apparatus. Therefore, I use the normal rectangular tank (breeding tank) as a behavioral test tank, hereafter.

In most cases, medaka males remained near the female without performing an apparent quick-circle (i.e., the male's courtship display) and interrupted the rival male without expressing aggressive behavior such as attack or bite (Kagawa. 2013; Kagawa 2014). Thus, a unique behavioral repertoire in the male-male competition emerges in the triad relationship.

Behavioral properties of mate-guarding behavior in medaka fish

Mate-guarding in most species is considered to be a male-specific behavior in conspecific social groups (Frankino et al., 1994; Tuni C et al., 2013; Beecher et al., 1979; Pinxten et al., 1997; Sherman, 1989; Tutin CEG, 1979; Buss DM, 2002). Extended periods of mate-guarding (pre- or post-spawning) differ among species (Frankino et al., 1994; Tuni C et al., 2013; Beecher et al., 1979; Pinxten et al., 1997; Sherman, 1989; Tutin CEG, 1979; Buss DM, 2002) . Here I investigated whether medaka fish exhibit the behavioral properties of mate-guarding. First, I examined whether medaka fish exhibit pre- or post-spawning mate-guarding. Sexually mature females have a 24-h reproductive cycle and spawn eggs once each morning (Fukamachi et al., 2009; Ono et al., 1957; Okuyama et al., 2014; Kobayashi et al., 2012). I compared the guarding indices of the near male (guarding test) just before and after spawning in the morning (Figures 4A and B). In addition, I performed the same test in the evening (Figure 4G) to examine whether males exhibit mate-guarding in a time of day-dependent manner. All three indices (62.6% ± 4.6%, 62.3% ± 3.7%, and 57.9% ± 6.1%, respectively) were significantly higher than the negative control (merged data: 30.4% ± 4.7%) (Figure 4H), indicating that mate-guarding occurred irrespective of spawning.

I then examined whether mate-guarding emerged in the presence of females of other fish species. When the medaka female was replaced with a female zebrafish (Figure 4C), the guarding index of the near male (31.9% ± 3.5%) was essentially the same as that of the merged control (30.4% ± 4.7%), suggesting that mate-guarding behavior is mediated by conspecific social cognition (Figure 4H). Next I examined whether two medaka females exhibit mate-guarding toward a male (Figure 4D). When two females and one male were placed in a single tank, there was not significant difference between the guarding index of the

near female between the two females (21.6% ± 2.9%) and that of the merged control (30.4% ± 4.7%) (Figure 4H). Furthermore, medaka males did not exhibit mate-guarding toward a male (Figures 4E and Figure 4H), and medaka females did not exhibit mate-guarding toward a female (Figures 4F and 4H). In addition, medaka males did not exhibit significant mate-guarding toward sexually-immature females (Figure 4H). Taken together, these results suggested that mate-guarding in medaka is a male-specific behavior toward sexually mature females.

Finally, I examined whether visual information is required for mate-guarding behavior. In medaka fish, social recognition in mating behaviors is mainly mediated by visual information (Okuyama et al., 2014). The guarding index of near males with a single eye removed was significantly lower than that of near males with sham ablation (Figure 5A). Single eye-removed males exhibit normal courtship behaviors, and thus the eye-ablation surgeries are assumed to not effect overall activity (Okuyama et al., 2014). In addition, medaka males exhibit significant mate-guarding toward females kept within a transparent cylinder tank that allows the males to only see the female without water intercirculating between the female's enclosure and the male's enclosure (Figure 5B and C). Taken together, these findings suggest that visual sensory information is necessary and sufficient to elicit male mate-guarding, although the possibility that other sensory information (pheromones, touch) modulates this behavior as well could not be excluded.

Positive correlation between dominance in mate-guarding and reproductive success

In various species, mate-guarding increases male reproductive success (Sherman, 1989; Komdeur, 2001; Komdeur et al., 2007). I examined whether dominance in

mate-guarding positively correlates with male reproductive success. In the present study, I designed a "dominance test" to determine which male was dominant based on the guarding index. Figure 6 shows a schema of the procedure used to determine the dominance of two males with different genotypes (A and B) in a triangle relationship. First, I measured the guarding index of a male with genotype A by calculating its relative position as described above. Then I measured the guarding index of a male with genotype B as the focal fish, and compared the "guarding indices" of two males with genotypes A and B (Figures 6 and 7).

To examine whether a dominant male had high reproductive success, I performed a paternity test using two males with different genotypes (one wild-type and the other a Tg expressing green fluorescent protein [GFP] in the primordial germ cells [*homozygote olvas*:*gfp*]). GFP detection in the medaka embryo allowed us to genotype the progeny. The day before mating, I performed a dominance test using wild-type and Tg males, and the next morning I performed a paternity test on the fertilized eggs from the females (Figure 2B). Medaka females have a single brood of 5 to 20 eggs each morning and, in most cases, the eggs are fertilized by the first male that exhibits synchronized copulation. Thus, I could determine which male won the competition for mating based on the paternity test (Okuyama et al., 2014). I performed a dominance test using 17 groups for 6 days (1 test/day; Figure 8). When Tg males were judged to be dominant (5/17 groups), the percentage of Tg progeny was approximately 93.6%. When wild-type males were judged to be dominant (7/17 groups), the percentage of Tg progeny was approximately 5.7%. In the remaining 5 of the 17 groups, I could not determine dominance because there was no significant difference in guarding index between the two males. The combined results of the dominance test and paternity test revealed that the dominant male had a significantly higher reproductive success rate than the

subordinate male (Figure 9).

Discussion

Here I showed that mate presence drives male competitive motivation, leading to mate-guarding behaviors in a triadic relationship. The behavioral repertoire of the triadic relationship, however, differs between courtship behavior in a dyadic setup and aggressive behavior elicited in a male group. Mate presence in several species facilitates male aggression (Jonart et al., 2007; Wells, 1988; Buena et al., 2008; Ancona et al., 2010). For examples, males in systems characterized by intrasexual competition (or females in some species, such as jacanas (Butchart et al., 1999) or syngnathid fishes (Paczolt et al., 2010)) require mechanisms that enable selective attention to females. Aggressive behaviors, however, were not enhanced in the triadic relationship of medaka fish. Thus, the unique behavioral repertoires (approach behavior to a mating partner and interrupting behaviors to a rival male) emerge in the triad relationship in medaka. In addition, I judged that this unique behavior can be considered as mate-guarding behavior in medaka, because dominance in this behavior increased reproductive success.

The present study demonstrated that medaka males exhibit mate-guarding behavior irrespective of the reproductive condition of the female, which is considered an exceptional example. In most species, males exhibit mate-guarding only during breeding periods. A long duration of mate-guarding is generally thought to lead to high energy cost and thus reduced male fitness (Parker, 1974). One possible explanation for the adaption of long-duration mate guarding in medaka is that the male behavioral trait increases male mating success via female preference. Medaka females prefer to mate with visually-familiar males that the female has seen for long time (~6 h). Thus, it is possible that males compete with each other to obtain

more visual contact with a potential mating partner to increase their reproductive success by enhancing female preference.

To my knowledge, this is the first report of mate-guarding behavior in a triad arrangement under laboratory conditions. By establishing a behavioral test to assess mate-guarding in a model organism, I was able to analyze the molecular basis underlying mate-guarding (Chapter 2).

Chapter 2

Analyses of the molecular basis underlying mate-guarding in medaka using VT-related gene mutants.

Introduction

In Chapter 1, I established a behavioral assay system to assess mate-guarding behavior in medaka. In this chapter, I investigated whether vasotocin (VT), a non-mammalian homolog of vasopressin (VP), is involved in mate-guarding in medaka.

In monogamous male prairie voles, the VP system is involved in mating-induced selective aggression toward a non-mate in a dyad, which may represent mate-guarding and be required to form pair bonding in the monogamous system (Lim et al., 2004; Winslow et al., 1993). In teleost fish, VT is implicated in various kinds of social behaviors, such as territorial behavior in a tropical damselfish (Santangelo et al., 2006; Santangelo et al., 2010) and pair bonding in a monogamous cichlid fish (Oldfield et al., 2011). In particular, VT increases both courtship and territorial behaviors of non-territorial males in the bluehead wrasse in the field (Semsar et al., 2001), implying that the VT system has an important role in the motivation of mating-related behaviors. The possible involvement of the VT/VP system in mate-guarding in a triad relationship, however, has not been investigated in these species under laboratory conditions. To evaluate the requirement of the molecular components of the VT pathways in the regulation of mate-guarding, loss of function analysis using gene KO animals is a valid and feasible method. Therefore, I generated medaka *vt* and V1a type vasotocin receptor 1 (*V1a1*) and V1a type vasotocin receptor 2 (*V1a2*) mutants using advanced molecular genetics, such as the TILLING (Targeting Induced Local Lesions IN Genomes) (Ishikawa et al., 2010; Taniguchi et al., 2006) and TALEN (Transcription Activator-Like Effector Nucleases) methods (Ansai et al., 2013; Ansai et al., 2014), and examined how the VT pathway is involved in mate-guarding behaviors.

Mate-guarding is presumed to be mediated by two different types of motivation: sexual motivation toward the opposite sex and competitive motivation toward the same sex. Therefore, I also checked dyadic behaviors, such as courtship behavior and aggressive behavior, of the mutants. These experiments allowed me to examine whether the motivation for mate-guarding is derived from 1) sexual motivation, 2) competitive motivation, 3) both sexual motivation and competitive motivation, or 4) neither sexual motivation nor competitive motivation.

Materials & Methods

Fish and breeding conditions

Medaka fish (*Oryzias latipes*; drR (the background strain of TALEN mutants), Cab (the background strain of TILLING mutants)) were maintained as described (Chapter 1).

Manning compound administration

This procedure was performed as previously described (Semsar et al., 2001) with minor modifications. After the guarding test, I anesthetized the near males using MS-222 and intraperitoneally injected the Manning compound. I injected 3.2 μg Manning compound/g body weight.

TILLING and high-resolution melting curve experiments

This procedure was performed as previously described (Ishikawa et al., 2010). To amplify the *vt*, *V1a1*, and *V1a2* locus that includes the final gene product, I performed polymerase chain reaction (PCR) with *vt*-specific primers: 5’ - AGACGTCCACACCGACA-3’ and 5’ - GCCAAAAGCATCTCACCT-3’, and *V1a1*-specific primers: 5’ - GGACAGCCTTTGCAACTT -3’ and 5’ - GTTTGTGGAGGAGAGGGTA -3’, and *V1a2*-specific primers: 5’ - CAGCGTGCTGCTCTTGA -3’ and 5’ - CGATGTAACGGTCCAAAGT -3’. The PCR conditions were as follows: 1 cycle of 94°C for 2 min, followed by 45 cycles of 94°C for 15 s; annealing at 63°C (*vt*) or 60°C (*V1a1, V1a2*) for 30 s, and then at 68°C for 30 s (*vt, V1a2*) or 60 s (*V1a1*); and a final denaturing and re-annealing step (1 cycle of 94°C for 30 s, followed by rapid cooling to 28°C). Each of the

5771 PCR products derived from genomic DNAs was subjected to the high-resolution melting assay. Based on differences in the melting curves, mutant candidates were selected. Melting curves were analyzed using the LightScanner (Idaho Technology, Salt Lake City, UT, USA), as previously described (Ishikawa et al., 2010). The mutations were then identified by sequencing the PCR product of the second positive genomic DNAs using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA) and the ABI 3730XL sequencing platform. I backcrossed the TILLING mutants with cab fish three times and crossed those fish to generate the homozygote mutants.

5' RACE of *vt*

Total RNA was extracted from the male medaka brain (drR and cab strains) using TRIZOL Reagent. 5'-Rapid amplification of cDNA ends (5'-RACE) was performed using a SMARTer RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. The amplification was performed using the Universal Primer A mix and 5'-TGATCCCAGCCTCCGGCAAT -3' (*vt* gene specific primer). The amplified PCR products were cloned into a pGEMT Easy Vector (Promega) and sequenced. I sequenced 10 cDNA clones derived from the drR strain and 9 cDNA clones derived from the cab strain and confirmed that the sequences of all 19 cDNA clones started from the transcription initiation site, which was predicted based on the annotated *vt* sequence.

Mass spectrometry (MALDI-TOF MS and SRM analysis)

Mass spectrometry (MS) was performed as described previously with minor modifications (Takemori et al., 2014; Takemori et al., 2009). Peptides in the pituitary of wild-type (Cab) and

*v*t mutant strains were extracted using 0.1% (v/v) trifluoroacetic acid (TFA). After concentration and purification with a self-made C18 STAGE tip (Rappsilber et al., 2003; Takemori et al., 2014), 5 μl of sample/one pituitary in 0.1% (v/v) TFA solution was analyzed using MS. To create peptide profiles of the pituitary of wild-type (Cab) and *v*t mutant strains, we performed peptide mass fingerprinting analysis using AXIMA TOF^2 MALDI-TOF MS (Shimadzu Biotech, Kyoto, Japan) (Suehiro et al., 2009; Suehiro et al., 2010). The peptide solution (0.5 uL) was mixed with 0.5 uL matrix solution [2% (w/v) α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile/0.1% (v/v) TFA] and spotted on a stainless-steel MS sample plate. To confirm the expression level of the avs peptide, SRM analysis was performed on a QTRAP 5500 coupled to an Eksigent nanoLC-Ultra system via a cHiPLC-nanoflex module (AB-SCIEX). Peptides were separated on a nano cHiPLC C18-reversed phase column (Chrome XP C18CL, 75 µm ID × 15 cm) and eluted at a constant flow rate of 300 nL/min. A linear gradient (2%–50% mobile phase B) was applied for 15 min, followed by a 6-min wash with 90% mobile phase B; the column was then equilibrated for 20 min with 2% mobile phase B.

TALEN experiment

TALEN experiments were performed as described previously (Ansai et al., 2013; Ansai et al., 2014). Potential TALEN target sites in the locus were searched using the TALEN Targeter program (https://tale-nt.cac.cornell.edu/node/add/talen). TAL repeats were assembled using the Golden Gate assembly method with slight modifications. Expression vectors for the TALENs were linearized by digestion with *NotI*. Capped RNAs were synthesized using the mMessage mMachine SP6 Kit (Life Technologies, Gaithersburg, MD, USA) and purified

using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Pairs of RNA for the TALENs (150 ng/μl) were injected into fertilized eggs of the drR strain by a microinjection method.

Prediction of secondary structure of V1a receptors

I obtained the genomic sequence information of V1a receptors from the Ensemble medaka genome browser (http://www.ensembl.org/Oryzias_latipes/Info/Index) and predicted their secondary structure using "SOSUI", which is a program that predicts transmembrane regions from amino acid sequences (http://bp.nuap.nagoya-u.ac.jp/sosui/).

Visual response and locomotion ability test (Optomotor response)

I assessed the optomotor response of the $vt^{M1R/M1R}$, $V1a2^{+/N68I}$, and $V1a2^{N68I/N68I}$ fish using our previously described apparatus (Imada et al., 2010) (see Figure 18). The medaka were placed in a fixed 15-cm-diameter circular tank, which was placed within a striped 20-cm-diameter cylinder. The depth of water in the tank was 2 cm. The striped cylinder was positioned on a rotatable metal disk that was driven by a motor, IHT6P3 (SERVO, Kiryu, Japan), that could move in either direction and at various speeds using the C-30PN (SERVO) motor driver. I recorded the optomotor response of medaka using a CCD camera (XC-ST70; SONY, Tokyo, Japan) and extracted the position of the medaka and stripes. A series of frames was analyzed using the software Move-tr/2D 7.0 (Library, Tokyo, Japan).

Courtship behavior assay

This procedure was performed as previously described (Okuyama et al., 2014). Males and

females were separated in the evening (18:00 - 19:00) the day before the assay. The mating pair was then placed together in a single tank (the light intensity was about 600-700 lx) the next morning, and mating behavior was recorded for 5 min. I counted the number of courtship displays. I performed a quality check on female reproductive states following our previously described procedure (Okuyama et al., 2014). I determined whether the females had spawned fertilized eggs 30 min after recording the movie. If the females had not produced fertilized eggs at that time, I judged that the females were not in a reproductive state, which might be due to stress or lack of food. The percentage of females in a reproductive state was 65%~75% and we did not analyze the data of the females not in the reproductive state.

Aggressive behavior assay

This behavioral assay was performed as previously described (Kagawa 2013; Kagawa 2014) with minor modifications. I placed three males of the same strain into a single tank (12 cm x 13 cm x 19 cm. Water depth was about 7-8cm, the light intensity was about 600-700 lx) and allowed them to adapt to the apparatus for 60~70min. Movement of each fish was recorded for 5 min. I defined two behavioral components, "bite" and "attack" (Kagawa 2013; Kagawa 2014) as "aggressive behaviors". The difference between "aggressive behaviors" in the previous work (Kagawa 2013; Kagawa 2014) was that in the present study we did not consider approaching and threatening behaviors that did not include touching each other (chase, replace, and frontal–lateral display) as "aggressive behaviors", because it is very difficult to discriminate these behaviors from shoaling-like behavior that *V1a2* mutants frequently exhibit in the male group. We counted aggressive behaviors of three fish (Figures 22, 24) or the focal fish (Figure 25).

Results

Essential role of V1a2 receptors for the emergence of mate-guarding

In prairie voles, the VP system is suggested to be involved in mate-guarding, because VP system mediates mate-induced selective aggression in a dyadic situation (Lim et al., 2004; Winslow et al; 1993). Therefore, at first, I focused on the VT as a candidate factor which regulated mate-guarding behavior in a triadic relationship and examined whether VT was involved in mate-guarding behavior of medaka fish using pharmacological methods.

Here, I found that injection of an VT antagonist (Manning compound) impaired medaka mate-guarding in the triad condition. The Manning compound is a commonly used antagonist of V1a receptors, including both subtypes V1a1 and V1a2 (Lema et al., 2004; Manning et al., 2008). I performed the guarding test using two males and one female, and then intraperitoneally injected an VT antagonist or saline into the near male. At 5 min after the injection, I performed a second test using the same trio of fish. The guarding index of the injected fish in the second test was significantly lower than that in the first test (Figures 10A and B). In addition, the guarding index of the uninjected males significantly increased 5 min in the second test (Figure 10C), while there was no effect of saline injection (Figure 10D). The VT antagonist did not affect the overall activity of the injected males (Figure 10E). This effect disappeared within 1 day after VT antagonist administration. These findings suggested that VT positively affected mate-guarding.

I then examined the possible involvement of individual molecular components in the VT pathway in mate-guarding by generating medaka mutants for genes encoding VT (Suehiro et al., 2009; Kawabata et al., 2012) and its receptors (V1a1 and V1a2)(Lema et al.,

2012). In fish species, two V1a-type receptors (V1a1 and V1a2) genes are expressed mainly in the brain (Lema et al., 2012; Iwasaki et al., 2013; Huffman et al., 2012; Kline et al., 2011) , while V2-type receptors are prominently expressed in tissues other than the brain, such as gills, heart, and kidney (Lema, 2010). I performed mutant screening for the mutants of *vt*, *V1a1*, *V1a2* from TILLING library: TILLING method is briefly summarized in Figure 11. TILLING library is composed of sets of frozen sperm and genomic DNA which were prepared from 5771 F1 males generated by wild-type females and N-nitroso-N-ethylurea (ENU)-mutagenized males (Taniguchi et al., 2006). I amplified specific loci of each genes using PCR, and identified mutations in PCR products by HRM (high-resolution melting curve) analysis and sequencing. Using this method, I identified *vt* mutants ($vt^{M1R/M1R}$) in which the first methionine residue was changed to arginine, *V1a1* mutants ($V1a1^{F93Y/F93Y}$) in which a conserved phenylalanine residue was changed to tyrosine, and *V1a2* mutants ($V1a2^{N68I/N68I}$) in which a conserved asparagine residue was changed to isoleucine (Figures 12-16). I confirmed that the 5' untranslated region of the annotated *vt* transcripts was identical with that determined by 5' Race method (Figure 12B). In addition, I performed mass spectrometry analysis to show that there was no detectable VT peptide in the brain of *vt* mutant (Figure 13). To examine whether mate-guarding emerges between two mutant males with the same genotype, I performed the mate-guarding test. The mate-guarding test using the wild-type males (Cab strain) that were used for generating the mutants indicated a guarding index of 49.2% ± 3.1 % for the near male, which was significantly higher than that of the merged control (35.3 % ± 1.5% merge). The guarding index of the wild-type males was relatively lower than that in previous experiments (Figure 4H), implying that external factors such as seasonal changes may affect my measurement of the guarding index of wild-type males.

Interestingly the guarding indices of the near male of the $vt^{M1R/M1R}$ and $V1a1^{F93Y/F93Y}$ mutants (43.7% ± 2.1% and 54.0% ± 3.0%, respectively) were significantly higher than that of the merged controls, indicating that mate-guarding emerges between these mutant males (Figure 17A). Thus, these two genes (*vt* and *V1a1*) were not required to elicit mate-guarding, suggesting that other ligand possibly activating the V1a2 receptor could function or compensate the deficit of *v*t. In contrast, there was no significant difference between the guarding indices of the near male among the $V1a2^{N68I/N68I}$ mutants (38.1% ± 2.3%) and that of the merged control (Figure 17A), indicating that mate-guarding did not occur between two *V1a2* mutant males. The $V1a2^{N68I/N68I}$ mutation did not affect overall activity and visual locomotion of the males (Figures 17B and 18). Thus, the V1a2 gene was required to elicit mate-guarding behavior. To confirm the behavioral phenotype of the loss-of-function mutations for the V1a1 and V1a2 genes, we generated *V1a1* and *V1a2* KO mutant males using the TALEN method (Figures 19 and 20A). The *V1a1* and *V1a2* KOs have 4 and 7-bp deletions in the first exon, respectively. Both of the mutated transcripts encode C-terminal deleted proteins, lacking at least six of the seven transmembrane domains encoded in the first exon. Considering that the V1a1 and V1a2 receptors are seven-transmembrane receptors, the lack of six of the transmembrane domains should lead to a loss of function. The guarding index of the near male of the *V1a1* KO mutant (49.6% ± 2.7%) was significantly higher than that of the merged control. In contrast, there was no significant difference between the guarding index of the near male of the *V1a2* KO mutant (37.3% ± 3.8%) and that of the merged control (Figure 20B). Thus, these findings further supported that the loss of function of the V1a2 gene, but not the V1a1 gene, attenuated mate guarding.

Essential role of the VT ligand for dominance of mate-guarding

I then examined whether these mutations affect dominance of male-male competition in mate-guarding behavior. I performed dominance tests using two male siblings with a different genotype: one a homozygote and the other a heterozygote mutant. The guarding index of $vt^{M1R/M1R}$ (23.0% ± 3.5%) was significantly lower than that of heterozygote mutant (38.9% ± 3.6%), and that of $V1a2^{N68I/N68I}$ (18.6% ± 3.9%) was significantly lower than that of heterozygote mutant (55.6% ± 6.4%; Figure 21A), indicating that $vt^{M1R/M1R}$ and $V1a2^{N68I/N68I}$ mutant males tended to be subordinate in male-male competition against their heterozygote mutant siblings. I also confirmed that the probability of greater proximity to the female of $vt^{M1R/M1R}$ and $V1a2^{N68I/N68I}$ males was significantly lower than that of heterozygote mutants (Figure 21B-D). In contrast, the guarding index of $V1a1^{F93Y/F93Y}$ (34.9 ± 4.0%) did not differ significantly from that of $V1a1^{+/F93Y}$ (32.5 ± 4.1%; Figure 21A), indicating that $V1a1^{F93Y/F93Y}$ mutant males show equivalent mate-guarding as the heterozygote mutants. These results demonstrated that *vt* homozygote mutants exhibited decreased dominance against *vt* heterozygote mutants in mate-guarding, although male-male competition for mate-guarding occurred between two *vt* homozygote mutant males (Figure 17A). Taken together, these findings suggest that VT ligands enhance dominance of male-male competition in mate-guarding.

Male sexual motivation toward the opposite sex and competitive motivation toward the same sex in the mutants for VT system

I examined whether these mutants have defects in sexual motivation toward the opposite sex and/or male competitive motivation toward the same sex that could cause

mate-guarding abnormalities. To test this issue, I tested aggressive behavior elicited in groups comprising only males and male courtship behaviors in a male/female pair, respectively. The homozygote *vt* mutant males exhibited normal aggression in a non-mate guarding situation (Figure 22A), whereas the mutant males exhibited fewer courtship displays than wild-type males (Figure 22B), showing that *vt* mutants normally have competitive motivation to the same sex (rival males), but they have low sexual motivation to the opposite sex (a potential mating partner) (Figure 23B). In contrast, the frequencies of aggressive behaviors and courtship display of homozygote *V1a2* mutant ($V1a2^{N68I/N68I}$) males were significantly lower than those of the wild-type control (Figure 22), indicating that the homozygote *V1a2* mutant did not normally have social motivation to either the same sex or opposite sex (Figure 23D). Interestingly, the frequencies of aggressive behaviors of heterozygote *V1a2* mutant ($V1a2^{+/N68I}$) males were significantly lower than those of the wild-type control (Figure 22A), while the heterozygote mutant males normally exhibited courtship displays (Figure 22B), revealing that the heterozygote mutant males have normal sexual motivation, but not competitive motivation (Figure 23C). The single functional *V1a2* allele might not produce enough of a gene product, leading to an attenuated aggression in a non-mate guarding situation. In conclusion, *vt* mutant males displayed defects in courtship display and did not normally exhibit mate-guarding (Figure 23B). In contrast, mate-guarding behavior exhibited by heterozygote *V1a2* mutant males appeared normal despite defects in aggression (Figure 23C). Based on these findings, the mate-guarding deficits of *vt* mutants were due to impaired sexual motivation, but not to impaired competitive motivation toward the same sex. In addition, behavioral analysis of *V1a1* mutant males suggested that V1a1 receptors are not required for either courtship or aggressive behavior (Figure 24), further implying a functional

difference between V1a1 and V1a2 receptors in social behaviors (Table 1). In addition, VT administered to wild-type males and *V1a2* heterozygote mutant increased the frequency of aggressive behaviors in a male group, but had no effect in *V1a2* homozygote mutant. Considering that *vt* mutants exhibit normal aggressive behaviors in a non-mate guarding situation, administration of exogenous VT might artificially activate V1a2 receptors, which enhances male aggression (Figure 25).

Discussion

In this Chapter, I analyzed mate-guarding behavior, courtship behavior and aggressive behavior of mutant males for VT system. The behavioral phenotypes in mate-guarding and aggressive behavior differed between the *vt* and *V1a2* mutants (Figure 23B and D). *V1a2* was required for the emergence of mate-guarding among mutant males, whereas *vt* was not required (Figure 17C). *vt* was required just for the dominance of mate-guarding (Figure 17D). The double functional *V1a2* allele was required to elicit aggressive behavior to the same degree as the wild-type, while the *vt* mutation did not significantly affect this behavior (Figure 22A). Only one gene encoding VT has been found in medaka genome based on genomic and transcriptome analyses (Kawabata et al., 2012), although the teleost genome is duplicated. These findings strongly suggested that V1a2 receptors are better conserved and more central in these signaling systems than the ligands and that some compensatory systems activate the V1a2 receptors in *vt* mutants. VT is not the only ligand that activates the V1a2 receptors (Figure 23). The involvement of VT/VP ligands in aggressive behaviors has been investigated based on pharmacologic manipulations in various vertebrates from fish to rodents (Santangelo et al., 2006; Santangelo et al., 2010; Semsar et al., 2001; Godwin et al., 2012; Haller et al., 2013; Fodor et al., 2014). Administration of exogenous ligands (VT) into the brain enhances aggression, while VT/VP receptor V1a antagonist suppresses aggression. In medaka, VT administration also enhanced aggression (Figure 25). These findings based on pharmacological analysis, however, do not preclude the possibility that other endogenous ligands activate the V1a receptors. These findings are consistent with a recent rodent study using a rat natural mutant with vasopressin (VP) deficiency (Fodor et al., 2014), which

revealed that a loss of function of the VP gene does not affect aggressiveness, especially in reproductively experienced males. Thus, the differences in behavioral phenotypes between *vt* and *V1a2* mutants may be due to the existence of redundant systems that activate the V1a2 receptors in medaka fish brain. Isotocin (IT), a non-mammalian homolog of OT, is a candidate endogenous ligand that activates V1a2 receptors. IT has affinity for the teleost VT receptor (Mahlmann et al., 1994) and there is significant cross-talk between OT, VP and their receptors in mammals (Song et al., 2014). Therefore, I also generated IT-related gene mutants and performed behavioral assay. I describe about it in the next Chapter (Chapter3).

My findings suggest that the VT system is involved in the process in which mate presence drives sexual motivation towards the opposite sex, which facilitates male competitive motivation for mate guarding in the triad relationship (Figure 23A). My finding is consistent with previous studies in the bluehead wrasse (*Thalassoma bifasciatum*) and African cichlid (*Astatotilapia burtoni*) (Huffman et al., 2014; Semsar et al., 2001). Pharmacologic manipulations of the VT system in both of the two fish species facilitates male courtship and territorial aggression, behaviors that are associated with social dominance (Oldfield et al., 2011; Kline et al., 2011). In non-fish species such as bird and frog, involvement of VT system in courtship and territorial aggression also has been suggested (Goodson,1998; Goodson et al., 2012; Penna et al., 1992). In mammalian species, the socially monogamous prairie vole (*Microtus ochrogaster*) has been used as a model organism for investigating the neurobiology of this type of complex social behavior, such as pair-bonding, which involves both intersexual and intrasexual interactions (McGraw et al., 2010; Young LJ et al., 2004; Donaldson et al., 2010). Prairie voles exhibit pair bonding behavior involving affiliation toward a mate and agonistic behavior toward non-mates. A series of studies using prairie voles revealed that VP

and the V1a receptor subtype have essential roles in pair bonding and other behaviors associated with monogamy (Lim et al., 2004; Winslow et al., 1993; McGraw et al., 2010; Young LJ et al., 2004; Donaldson et al., 2010). In the monogamous vole, however, each of the behavioral components (affiliation toward mates and agonistic behavior toward non-mates) was analyzed individually in the dyad setup (McGraw et al., 2010; Young LJ et al., 2004; Donaldson et al., 2010), and possible involvement of the VT/VP system in the actual mate-guarding behavior in the trial relationship has not yet been investigated in prairie voles. Establishing a quantitative assay system for this behavior under laboratory conditions allowed us to genetically study the molecular mechanisms underlying mate-guarding behavior in a triad experimental setup, but not dissect the sub-behaviors, which have been studied for several decades.

Furthermore, it should be noted that the VT receptor V1a2 has a central role in regulating fish social behaviors such as mate guarding, courtship behaviors, and aggressive behaviors. The VT receptor function in fish species has been investigated based on pharmacologic manipulations alone, and the selectivity of mammalian V1a receptor antagonists like the Manning compound for the two V1a-type receptors (V1a1 and V1a2), is unknown (Santagelo et al., 2006; Santagelo et al., 2010; Oldfield et al., 2011; Semsar et al., 2001; Manning et al., 2008; Mahlmann et al., 1994). Thus, functional differences between the two receptors cannot be determined based on pharmacologic studies alone. Some recent studies reported differential gene expression between *V1a1* and *V1a2*. In the larval brain of zebrafish, the two genes are expressed in the same brain regions, but few neurons coexpress *V1a1* and *V1a2* (Iwasaki et al., 2013). The differential regulation of gene expression levels in the bluehead wrasse *Thalassoma bifasciatum* implies the importance of V1a2 over V1a1 in

fish social behavior (Lema et al., 2012). My findings suggested differences in the behavioral function between V1a1 and V1a2 in mate-guarding.

In adult fish brains, *V1a2*-expressing neurons are broadly located in various brain regions, such as the dorsal and ventral telencephalon, and the preoptic area (Lema et al., 2012; Iwasaki et al., 2013; Kline et al., 2011), that are thought to be important for social motivation (O'connell et al., 2011). It remains unknown, however, how *V1a2*-expressing neurons mediate individual social components in different social contexts. More importantly, it should be noted that the gene KO method eliminates VT or its receptors in the relevant social behavior-related neural areas, but also more widely in the brain and other tissues such as the gonads (which also express VT receptors) throughout development. Further studies are needed that selectively manipulate subpopulations of *V1a2*-expressing neurons in the adult brain. Genetic mosaic techniques are available in medaka fish to visualize and/or genetically modify a neuronal subpopulation within complex neural circuits (Okuyama et al., 2013; Suehiro et al., 2010). Genetic dissection of the VT system using such advanced molecular genetic methods will allow us to identify the microcircuits that regulate social behaviors. The present study using medaka mutants is an important first step toward unveiling this complex neuromodulatory pathway, for which our current understanding is very poor.

Chapter 3

Analyses of the molecular basis underlying mate-guarding in medaka using IT-related gene mutants

Introduction

In the previous chapter, I demonstrated that VT and V1a2 are required for normal mate-guarding behavior, but *V1a2* mutants exhibited more severe defects in triadic and dyadic behaviors than *vt* mutants. The differences in behavioral phenotypes between *vt* and *V1a2* mutants might be due to the existence of redundant systems that activate the V1a2 receptors in medaka fish brain. Isotocin (IT), a non-mammalian homolog of oxytocin (OT), is a candidate endogenous ligand that activates V1a2 receptors, because IT has affinity for the teleost VT receptor (Mahlmann et al., 1994) and there is cross-talk between OT, VP, and their receptors in mammals (Song et al., 2014).

In this chapter, I describe the behavioral phenotypes of mutants for the IT system gene. While there have been extensive studies of VT function in fish, only a few studies have examined the IT function in fish behavior. For example, IT administration stimulates approach behavior towards conspecifics in goldfish (*Carassius auratus*), whereas VT has the opposite effect (Thompson et al., 2004). IT and VT modulate vocalization of the plainfin midshipman *(P. notatus*) in non-territorial and territorial males, respectively (Goodson et al., 2000), implying that the IT system regulates fish social behaviors differently than does the VT system. Therefore, I generated medaka mutants for IT and isotocin receptor 1 (ITR1) genes using the TILLING and TALEN methods, and examined how the IT pathway is involved in mate-guarding behavior. In addition, I examined whether the IT-related gene mutation could affect dyadic relationships, such as courtship behavior and aggressive behavior, to evaluate whether sexual motivation and/or competitive motivation correlated with the mate-guarding motivation in the IT system.

Materials & Methods

Fish and breeding conditions

Medaka fish (*Oryzias latipes*, drR strain and Cab) were maintained as described (Chapter 1).

TILLING and high-resolution melting curve experiments

This procedure was performed as described (Chapter 2). To amplify the *it* locus that includes the final gene product, I performed polymerase chain reaction (PCR) with *it*-specific primers: 5’- TTTCTTCCTTTCTCTTCTTTTAATACC -3’ and 5’- TTCCAAATTCAAGGAAAGGCT -3’. The PCR conditions were as follows: 1 cycle of 94°C for 2 min, followed by 45 cycles of 94°C for 15 s; annealing at 58°C, and then at 68°C for 30 s; and a final denaturing and re-annealing step (1 cycle of 94°C for 30 s, followed by rapid cooling to 28°C).

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the medaka brain (male and female mixed drR strain) using TRIZOL Reagent. I performed reverse transcription using Primer Script RT reagent kit and this resulting cDNA was used as template for amplifying the *ITR1* and *ITR2* loci by PCR with *ITR1*-specific primers: 5’- CAATGAAAGTGAGATCTGGCAGTT -3’and 5’- CGATGCAAGGAGTGCAGAG -3’ and *ITR2*-specific primers: 5’- TACTTGAGCTGGAAAGTTGGTCT -3’ and 5’- CGACAGTGGATGAGTTCCTTT -3’. The PCR conditions were as follows: 1 cycle of 94°C for 2 min, followed by 30, 33 or 35 cycles of 94°C for 30 s; annealing at 58°C, and then at 72°C for 30 s (*ITR1*) or 50 s (*ITR2)*.

Prediction of secondary structure of ITR1

I obtained the genomic sequence information of *ITR1* from the Ensemble medaka genome browser (http://www.ensembl.org/Oryzias_latipes/Info/Index) and predicted their secondary structure using "SOSUI", which is a program that predicts transmembrane regions from amino acid sequences (http://bp.nuap.nagoya-u.ac.jp/sosui/).

Results

Facilitation of mate-guarding in it mutant males

To examine the possible involvement of IT systems in mate-guarding in medaka, I generated *it* mutants using the TILLING method. I identified *it* mutants ($it^{I22F/I22F}$) in which a conserved isoleucine residue was changed to phenylalanine (Figure 26). I performed the mate-guarding test to examine whether mate-guarding emerges between two mutant males with the same genotype. The guarding index of the near male of the $it^{I22F/I22F}$ mutant (57.1% ± 2.9%) was significantly higher than that of the merged controls (35.3% ± 1.5%), indicating that mate-guarding occurs between $it^{I22F/I22F}$ mutant males (Figure 27A). Thus, IT was not required to elicit mate-guarding.

Next, I examined whether the *it* mutation affects the dominance of male-male competition in mate-guarding behavior. I performed dominance tests using two male siblings with different genotypes: one a homozygote and the other a heterozygote mutant. The guarding index of $it^{I22F/I22F}$ (59.1% ± 6.7%) was significantly higher than that of heterozygote mutant (23.0% ± 4.7%; Figure 27B). I also confirmed that the probability of greater proximity to the female of *it* homozygote mutant males was significantly higher than that of heterozygote mutant males (Figure 27C). These results indicated that $it^{I22F/I22F}$ mutant males tended to be dominant in male-male competition against their heterozygote mutant siblings. This result is in contrast to that of $vt^{M1R/M1R}$, suggesting that IT ligands suppress dominance of male-male competition in mate-guarding in a triad.

Expression analysis of two genes for IT receptors (ITR1 and ITR2)

I then examined the target receptors of IT ligands regulating mate-guarding. Using the genomic database, I identified two subtypes of genes encoding IT receptors ITR1 and ITR2. At that time, however, there were no reports of differences in the expression patterns of these two genes. Therefore, I examined whether *ITR1* and/or *ITR2* was expressed in the medaka brain using RT-PCR, before I generated the KO mutants of the IT receptor genes. The PCR products derived from *ITR1* were detected at the predicted size (450 bp), while those from *ITR2* were not (Figure 28), suggesting that at least *ITR1* is expressed in the adult medaka brain.

Facilitation of mate-guarding in ITR1 KO mutant males

I generated *ITR1* KO mutants using the TALEN method. The *ITR1* KO mutants have a 7-bp deletion in the first exon (Figure 29). The structure of ITR1 is almost the same as those of V1a1 and V1a2 and the deletion mutation resulted in the loss of six of the transmembrane domains in *ITR1* KO mutants, which should lead to a loss of function. In the guarding test, the guarding index of the near male of the *ITR1* KO mutant (49.2% ± 5.2%) was significantly higher than that of the merged control (32.1% ± 2.3%; Figure 30A), indicating that *ITR1* was not required to elicit mate-guarding. Next, I performed dominance tests using *ITR1* KO mutant males and wild-type males. The guarding index of *ITR1* KO mutant males (41.2% ± 4.6%) was significantly higher than that of wild-type male (28.2% ± 3.8%; Figure 30B). I also confirmed that the probability of greater proximity to the female of *ITR1* KO mutant males was significantly higher than that of wild-type males (Figure 30C). The behavioral phenotype of *ITR1* mutant males was consistent with that of the *it* mutants,

strongly suggesting that IT ligands suppressed the dominance in mate-guarding via ITR1 activation.

Male sexual motivation toward the opposite sex and competitive motivation toward the same sex in IT system gene mutants

The findings of Chapter 2 suggested that the mate-guarding deficits of *vt* mutants were due to impaired sexual motivation, but not to impaired competitive motivation toward the same sex. Here I examined whether IT system gene mutants had enhanced sexual motivation toward the opposite sex and/or male competitive motivation toward the same sex that could facilitate mate-guarding. To evaluate this issue, I tested aggressive behavior and male courtship behaviors. Unexpectedly, the homozygote *it* mutant males exhibited normal aggression in a non-mate guarding situation and normal courtship displays (Figures 31A-B), showing that *it* mutants have normal competitive motivation and sexual motivation (Figure 32). I performed courtship behavior testing using only *ITR1* KO mutant males, because the drR strain, which is used to generate *ITR1* KO mutants by the TALEN method, exhibited much less aggression than the Cab strain used to generate *it* mutants by the TILLING method, which made it difficult to evaluate the aggressive behavior of the drR strain using same behavioral assay. The *ITR1* KO mutant males exhibited normal courtship behavior, indicating that the *ITR1* KO mutant males also have normal sexual motivation (Figure 31C). These findings implied that the facilitation of mate-guarding of *it* mutant males was not due to either enhanced sexual motivation or competitive motivation driven in the dyadic relationship. The IT system might be involved in regulating male social motivation enhanced only in the triadic relationship (i.e., the presence of both a rival and a mating partner).

Discussion

In this Chapter, I analyzed mate-guarding behavior, courtship behavior, and aggressive behavior of IT system gene mutant males. The behavioral phenotypes of *it* and *ITR1* mutants were almost the same, and the two types of mutants showed high dominance in mate-guarding and normal dyadic behavior, suggesting that the IT ligands activates ITR1, which mediates high dominance in mate-guarding. In addition, neither the VT nor IT ligands were required for normal aggression elicited in an all-male group. Given that there is cross-talk between IT, VT, and their receptors (Mahlmann et al., 1994), further analysis, such as double KO (*vt* and *it*), is required to investigate the possible involvement of VT and IT ligands in the aggressive behaviors elicited in all-male groups.

Two candidate IT/OT receptor subtypes have been reported only in medaka and zebrafish (Chou et al., 2011). A functional difference between the ITR1 and ITR2 has not been established. Here I showed that *ITR2* expression in the medaka brain was undetectable using RT-PCR, suggesting that the expression level of *ITR2* is very low. Based on an assumption that *ITR2* could function as an IT receptor, the similar behavioral phenotypes of the *it* and *ITR1* mutants suggested that *ITR1,* rather than *ITR2*, mainly functions in regulating mate-guarding.

Investigation of the behavioral function of mammalian OT and non-mammalian OT [mesotocin (MT, the avian and reptile homolog) and IT] systems, revealed that the OT systems could modulate various social processes, such as social recognition (Goodson et al., 2010) (See Chapter 4), bonding (Donaldson et al., 2008), affiliation (Insel, 2010), parental care (Pedersen et al., 1994), anxiolysis (Neumann, 2009), and appetite (Olszewski et al.,

2010) in mammals. Most of the previous studies revealed that OT systems regulate mating-related behaviors, especially in females, such as maternal and sexual behaviors (Young WS et al., 2003). Thus, the OT systems are generally believed to be involved in female-specific behaviors (Donaldson et al., 2008). For example, in the plainfin midshipman fish, female social fictive vocalization in courting is sensitive to IT and its antagonist, but not to VT and its antagonist. In males, the sensitivity is reversed (Goodson et al., 2000). In addition, in the monogamous prairie vole (*Microtus ochrogaster*), the OT system has an essential role in pair bonding only in the females, and OT administration does not affect pair bonding in males, while VP and the V1a receptor subtype have essential roles in pair bonding in the males, suggesting the possible involvement of the VP system in mate-guarding (Insel et al., 1998). My findings also suggest that the OT/MT/IT system does not regulate male specific dyadic behavior, such as courtship behavior and aggressive behavior. In mate-guarding induced by triadic relationships, however, the IT system is involved in regulating male specific behaviors. The novel behavioral test I developed to assess mate-guarding in triadic relationships sheds light on a new role of the OT/MT/IT system in male mating-related behavior.

In the present study, comparing behavioral phenotypes between dyadic and triadic behaviors also allowed me to address the role of the IT system in more detail. If I had only focused on the results of mate-guarding, my findings would suggest that the IT and VT systems, which suppress and promote mate-guarding, respectively, have opposite roles. This finding seemed to be consistent with previous studies in the goldfish (*Carassius auratus*) demonstrating that IT administration stimulates approach behavior towards conspecifics, whereas VT has the opposite effect (Thompson et al., 2004). Based on my behavioral analysis

of the dyadic relationship, however, I assumed that the opposite effect of VT and IT mutation in mate-guarding derives from abnormalities of different factors (Table 2). Taken together, the VT system might be involved in processes in which mate presence drives sexual motivation towards the opposite sex, which facilitates male competitive motivation for mate-guarding in the triad relationship (Chapter 2). In contrast, the IT system might be involved in the processes required only in the triadic relationship, which is different from the VT-mediated process. Therefore, the VT and IT systems may affect social behavior through independent processes, although VT and IT administration sometimes has opposite effects on the same behavior (Thompson et al., 2004; Goodson et al., 2000) and the expression patterns of the VT receptor and the IT receptor are somewhat overlapped in teleosts (Huffman et al., 2012).

In all vertebrates, OT/MT/IT peptides are produced by magnocellular and parvocellular neurons in the preoptic area (POA) and anterior hypothalamus (AH) (Goodson et al., 2010) and these neurons project to the pituitary, allowing the peptides to function broadly in various regions of body. Relative to the large number of studies of the distribution of OT/MT/IT ligands, there are only a few studies on the distribution of their receptors (Insel et al., 1992; Huffman et al., 2012; Leung et al., 2011). Although widespread expression in forebrain and midbrain is reported in some vertebrates, the functions of the OT/MT/IT receptor-expressing regions remain largely unknown (Huffman et al., 2012). Therefore, further studies are needed in which subpopulations of *ITR1*-expressing neurons are selectively manipulated in the adult brain, as described for the VT system in social behaviors in Chapter 2.

Chapter 4

Analyses of the molecular basis underlying social motivation according to social familiarity

Introduction

Some social animals can recognize socially-familiarized conspecific members (social recognition) and familiarity affects their social motivation, which may be important for social adaptation. Disorders in human brain function for social motivation are assumed to cause mental illnesses, such as autism, and great attention has recently been paid to the underlying neural/molecular mechanisms of these disorders. Oxytocin (OT) is considered to be involved in social recognition and motivation in rodents and humans (Bielsky et al., 2004; Wacker et al., 2012; Bartz et al., 2011). For example, intranasal injection of OT in humans improves social function in children with autism (Gordon et al., 2013). Social familiarity in rodents influences male mating behaviors. Social familiarization (repeated encounters) decreases approach behaviors of male wild-type mice toward unfamiliar females, but not OT KO males (Ferguson et al., 2000). Thus, the OT KO males are thought to have defects in social recognition (social amnesia).

In Chapters 1～3, I described the results of behavioral tests in which I used group-reared fish (defined as familiar fish) in the same tank for individual behavioral tests. In this chapter, I investigated social behaviors of individual medaka toward unfamiliar conspecifics reared in a different tank. In our recent publication (Okuyama et al., 2014), we clearly demonstrated that social familiarization affects medaka mating behaviors, especially in females. Medaka females tend to choose visually familiarized males as their mating partner. In contrast, wild-type males exhibit mating behaviors, irrespective of social familiarization (Okuyama et al., 2014). In the present study, I found that social familiarization strongly affects male social behaviors (e.g., courtship, aggressive, and mate-guarding behaviors) only

in mutants for IT-related genes, but not in wild-type males, suggesting that mutants for IT-related genes lost social motivation toward conspecifics.

In some fish, imprinting affects the social preferences of the juvenile fish based on the traits of their parents that care for them during the early development period (Verzijden & ten Cate 2007). In the present study, I used “familiar fish” as fish that were always reared together since birth. Next, to examine whether or not imprinting mediates social recognition of mutants for IT-related genes, I investigated whether social familiarization (rearing in the same tank only as adults) could recover the loss of social motivation in the mutants toward unfamiliar fish.

Materials & Methods

Fish and breeding conditions

Medaka fish (*Oryzias latipes*, drR strain, Cab strain, IT-related gene mutated fish and Tg(actin pro::red fluorescent protein [RFP])) were maintained as described in Chapter 1. Wild-type fish and each mutant fish were bred in different tanks (only *it* heterozygotes and *it* homozygotes were bred in the same tanks because they were born from the same parents).

Social familiarization for behavioral tests of courtship and mate-guarding behaviors

Four fish (2 focal males and 2 females) were reared in a single tank for social familiarization. In courtship behavior assay, two females were wild-type fish used as a mating partner, while two genetically marked (Tg) female fish, which express Red Fluorescent Protein (RFP), were used for a guarding target fish. The males and females were derived from two different tanks, respectively. Thus males and females were unfamiliar with each other before this procedure. I used identical fish before and after this procedure for individual behavioral tests, as "unfamiliar" fish and "socially-familiarized" fish, respectively.

Aggressive behavior assay

I followed the previous procedure described in Chapter 2, except for one modification. In the present experiment, I also used genetically-marked RFP males (Tg-fish), which allowed me to visually distinguish a focal male from the two other Tg males in the same tank. I counted aggressive behaviors of the focal fish toward the other two Tg-males. To exclude the possibility that a low number of aggressive behaviors in the focal fish derived from a low

expression of dominance of the three males, I only counted the tests in which the focal male exhibited the highest number of aggressive behaviors as a trial.

Social familiarization for behavioral tests of aggressive behavior

Four fish (2 focal fish males and 2 RFP males) were reared in a single tank for social familiarization. The focal males and RFP males were derived from the same tanks. Thus, males and females were unfamiliar with each other before this procedure. I used the identical Tg-fish before and after this procedure for individual behavioral tests, as unfamiliar fish and familiar fish, respectively.

Female mating preference

This behavioral assay was performed as previously described (Okuyama et al., 2014). Male and female medaka were fed brine shrimp (3 ~ 4 times per day) for 2 days before the mating behavior assay. The males and females were separated in the evening (18:00 to 19:00) on the day before the assay, using two methods (Group 1 and Group 2). In Group 1, the female was permitted to see the male prior to mating; the female was kept in a plastic aquarium (12 cm X 13 cm X 19 cm) under normal breeding conditions, while the male was separated from the female in a columnar glass aquarium (transparent cylinder with a radius of 5 cm, height 15 cm). In Group 2, to prevent visual contact of the male and female, the columnar glass aquarium was wrapped with opaque white paper. Before separation, surplus brine shrimp, algae, and debris were thoroughly removed from the aquarium, and water flow from the tank recycling system was stopped. The male was quietly introduced into the aquarium with the female in the morning for the mating behavior assay (10:00 to 12:00). The mating behavior of

the pair was recorded for 5 min using a digital video recorder. Based on the recording, the timing of male quick-circle dance performances and copulations followed by spawning by the pair were determined.

Results

Courtship behavior toward socially unfamiliar females

In mice, repeated meetings with the same ovariectomized female leads to decreased sniffing duration of wild-type males, but not OT KO males (Ferguson et al., 2000). To examine whether the medaka intersexual behaviors change according to the social familiarization, I compared the courtship behavior toward a "familiar female", which had been bred in the same tank with males since birth, and that toward an "unfamiliar female", which was bred in a different tank. In addition, to examine whether IT system is involved with its change as OT system in mice, I used wild-type males and *it* mutant males that were generated by the TILLING method (Chapter 3).

The number of courtships of wild-type toward socially unfamiliar females (*it* homozygote mutant) did not differ significantly from those toward familiar females (wild-type) (Figure 33), suggesting that wild-type males exhibit courtship behavior irrespective of social familiarity. In contrast, the number of courtships of *it* homozygote mutant males toward unfamiliar females (wild-type) was significantly lower than that of wild-type males (Figure 33), while *it* homozygote mutant males exhibited normal courtship behaviors toward familiar females (*it* homozygote mutant females; Chapter 3, Figure 33). This result suggested that *it* homozygote mutant males exhibit normal courtship behavior only toward socially familiar females, but not toward socially unfamiliar females. In this experiment, I used mutant males and females. Next, to examine whether this behavioral defect in *it* homozygote mutant males was due to the mutation in the focal male fish or the mating partner (female), I used wild-type females and mutant males for the courtship behavior assay.

In addition, to test whether the behavioral defect in *it* homozygote mutant males were recovered by social familiarization, I kept a pair (male and female) in the same tanks for a certain period of time and investigated the effect of the social familiarization on courtship behaviors. The number of courtship displays of *it* homozygote mutant males after 19-20 day social familiarization was significantly higher than that before social familiarization (Figure 34A), suggesting that 19-20 day social familiarization was sufficient for *it* homozygote mutant males to recover their sexual motivation toward socially-familiarized partners. Thereafter, I used fish after a 19-20 day social familiarization as "socially-familiarized" fish. I then performed a courtship behavior assay for socially-familiarized females using wild-type and *it* heterozygote mutant males. The numbers of courtships of these males towards unfamiliar females were not significantly different than those towards socially-familiarized females (Figure 34B). In addition, the number of courtship behaviors towards socially-familiarized females in those males and that in *it* homozygote mutant males was almost the same. These results supported that *it* homozygote mutant males had low sexual motivation only toward socially unfamiliar females and this low motivation was almost recovered to the normal level following a 19-20 day social familiarization. Taking into account that the results of the courtship behavior assay using *ITR1* KO males were almost the same as those using *it* homozygote mutant males (Figure 34C), IT might promote sexual motivation of males toward socially unfamiliar females via ITR1 activation.

Mate-guarding behavior toward unfamiliar and socially-familiarized females

I then examined whether IT-related gene mutant males also changed their mate-guarding behavior according to the social familiarity with females. First, I performed

the guarding test. When I used unfamiliar females, the guarding index of the near male of wild-type and *it* heterozygote mutants was significantly higher than that of the merged control (Figure 35A), suggesting that wild-type males exhibited mate-guarding behavior irrespective of the social familiarity of the females (Chapters 1-3). In contrast, in *it* homozygote mutant males and *ITR1* KO males, the guarding indices of the near male toward unfamiliar females was not significantly different than those of merged controls (Figures 35A-B). Moreover, social familiarization recovered this defect in mate-guarding behavior (Figures 35A-B). Taken together, these findings suggested that *it* and *ITR1* are required for mate-guarding behavior toward unfamiliar females.

I also performed the dominance test. As described in Chapter 3, mutant males for the IT system genes showed high dominance in the dominance test using socially familiar females (Figures 27B and 30B). When an unfamiliar female and two males (an *it* heterozygote mutant and a homozygote mutant or a wild-type fish and a *ITR1* KO mutant) were used, however, the guarding indices of the *it* homozygote mutants and *ITR1* KO mutants were significantly lower than those of the *it* heterozygote mutant and wild-type fish, respectively (Figure 36). These results supported that mutant males for IT system genes lack social motivation toward an unfamiliar female, which may lead to decreased competitive motivation for mate-guarding.

Aggressive behavior toward socially unfamiliar and familiarized males

I then examined whether social familiarity affected aggressive behavior in male groups of wild-type and *it* mutant males (I did not examine the aggressive behavior of *ITR1* KO mutant males for the reasons explained in Chapter 3). In Chapters 2 and 3, I used three

males with the same genetic background for the aggressive behavior assay and counted the total number of aggressive behaviors of the three fish. In this chapter, to compare the aggression toward unfamiliar males and that toward socially-familiarized males, I used one focal male and two RFP males (Tg(actin pro::mCherry)) and counted the aggressive behaviors of the focal male towards the RFP males (see Material & Methods). While wild-type and *it* heterozygote mutant males exhibited aggressive behaviors toward unfamiliar RFP males, *it* homozygote mutant males hardly exhibited aggressive behaviors toward them (Figure 37). In addition, this low competitive motivation in *it* homozygote mutant males was recovered by social familiarization with the RFP males (Figure 37A). When I used three males with the same genotype (Chapter 3), there were no significant differences between the numbers of aggressive behaviors of dominant (most aggressive) males in wild-type males and those in *it* heterozygote mutants and *it* homozygote mutants (Figure 37B), which suggests that IT is required to exhibit normal aggressive behavior toward socially unfamiliar males.

Female sexual preference in IT-related gene mutant females

As described above, medaka females tend to choose visually familiarized males as their mating partner and reject visually unfamiliar males. Therefore, I examined whether IT-related gene mutant females also showed abnormal social motivation according to social familiarity, as IT-related gene mutant males do. In this experiment, a long latency to mate means that the females rejected the males many times. While visual familiarization significantly decreased the latency to mate in wild-type and *it* heterozygote mutant females, in *it* homozygote and *ITR1* KO mutant females, the latency to mate with unfamiliar males was as short as those with visually familiarized males (Figure 38). Given that mutant females have

defects in social behavior toward unfamiliar fish, this behavioral phenotype in *it* homozygote and *ITR1* KO mutant females might be similar to that in mutant males.

Discussion

Here, I showed that IT system gene mutant fish had low social motivation toward unfamiliar fish in courtship, mate-guarding, and aggressive behaviors, but had normal or high motivation for those behaviors toward socially familiar fish. In mice, social familiarization decreases the sniffing duration of female odors in wild-type males, but not OT KO males. The sniffing duration in OT KO males was as long as before social familiarization (Ferguson et al., 2000). Therefore, in the mouse study, it was impossible to determine whether the continuous sniffing in the OT KO males was derived from abnormalities of social recognition or social motivation. Thus, it is generally interpreted that OT KO mice have defects in social recognition (learning and memory of other members) and cannot discriminate between socially unfamiliar and familiar individuals. In contrast, my findings of behavioral phenotypes of mutants for the IT system genes showed that those fish had no defect in the recognition ability, because they could discriminate between socially unfamiliar and familiar fish. It suggested that the IT/OT system regulates other process such as social motivation. Recently, great attention has been paid to the use of OT to treat people with autism, who tend to ignore people they meet for the first time (Gordon et al., 2013). Thus, autistics can distinguish between socially familiar and unfamiliar people. Therefore, it is plausible that the OT system does not regulate social recognition, but rather regulates social motivation in humans as IT system in medaka. I expect that further studies of IT system mutant medaka provide clues to the mechanisms underlying mental illness, such as autism, in humans.

Furthermore, I demonstrated that the low motivation toward socially unfamiliar fish in IT-related gene mutants was recovered by social familiarization in the adult stage. In some

fish, mate preference and sympatric speciation are affected by familiar individuals during early developmental stage (i.e., imprinting) (Verzijden & ten Cate 2007; Kozak et al., 2009). In contrast, exposure to females after sexual maturity affects to the mate choice of male guppies and males choose the females with different color type from exposed females (Haskins et al., 1950). My findings suggested that the social motivation according to social familiarity in IT-related gene mutants derived not from imprinting but from learning after sexual maturity and neural networks that regulate social behaviors remain plastic in the adult medaka brain.

General Discussion

In the present thesis, I found that medaka males exhibit mate-guarding behavior in triadic relationships and established novel behavioral assay system to quantify this behavior. To my knowledge, this is the first report of mate-guarding behavior in a triad relationship under laboratory conditions.

Further, using genetic methods, I showed that the VT system promoted mate-guarding behavior, whereas the IT system suppressed mate-guarding behavior (Chapters 2 and 3). My findings appeared to be consistent with previous findings in goldfish that VT and IT induced opposite effects on social approach behavior (Thompson et al., 2004). Additional behavioral analysis (courtship behavior and aggressive behavior) of these gene mutants in medaka, however, suggested that the VT and IT systems mediate mate-guarding via different pathways (Figure 40). Impaired mate-guarding in *vt* and *V1a2* homozygote mutants may be due to the loss of sexual motivation toward the opposite sex, and not to the loss of competitive motivation toward rival males. In contrast, the high dominance of *it* and *ITR1* KO mutants may be due not to dyadic motivation but rather to the social motivation specifically induced in a triad. In various vertebrates, the VP/VT and OT/MT/IT genes are located with reverse direction adjacent to each other in the same chromosome and specific regulatory cis-elements of VP/VT and OT/MT/IT genes locate in the intergenic region of these genes (IGR hypothesis) (Young WS et al., 2003). Some researchers, therefore, have proposed that the VP/VT and OT/MT/IT genes work in a coordinated manner (Stoop, 2012). The findings of the present thesis, however, suggested that VT and IT regulate mate-guarding via independent pathways and support the viewpoint that the VT and IT systems regulate the

same behavior via different pathways.

Mate-guarding behavior is considered part of male-male competition, and many studies have focused only on dyadic aggressive behavior in the presence of females (Lim et al., 2004; Winslow et al., 1993). In the present study, the novel behavioral test I developed to assess mate-guarding made it possible to discriminate the social motivation induced in dyadic relationships (male-male or male-female) from that in triadic relationships (2 males and 1 female). I then showed that mate-guarding behavior exhibited by heterozygote *V1a2* mutant males appeared normal despite defects in aggression. This result suggested that the neural mechanisms underlying the triadic relationship differ somewhat from those underlying male aggressive behaviors. Considering that mate-guarding requires simultaneous dual attention to both opposite and same sexes, brain information processing system regulating the triad relationship is assumed to be involved in the integration of sensory inputs from two different types of objects (opposite and same sexes) and simultaneous elicitation of affiliative behavior toward the opposite sex and agonistic behaviors toward the same sex. Further analysis is required to elucidate how the VT and IT systems are involved in the neural network intrinsic to triadic relationships.

Finally, I demonstrated that IT system gene mutants lost social motivation toward unfamiliar individuals in mate-guarding, and aggressive and courtship behaviors in males and rejecting behavior in females, while social familiarity had no effect on social motivation in wild-type males (Chapter 4). Researchers have recently begun to investigate how social familiarity affects social behaviors with human neuroimaging studies seeking to map higher-order brain processing of social information (Hari et al., 2009). Investigating the molecular mechanisms of the human brain using genetic methods, however, is generally

considered impossible. Thus, animal studies are required for dissecting the molecular mechanisms. Mice have been used to address this issue because social familiarization reduces some social behaviors, such as sniffing, toward unfamiliar individuals (Wacker et al., 2012). As described in Chapter 4, OT KO mice are thought to have "social amnesia" and impaired "learning and memory" for social recognition (Ferguson et al., 2000). Whether OT KO mice lose the ability for other social processes, such as social motivation, rather than "learning and memory" for social recognition, has not yet been determined, however, because these two possibilities cannot be discriminated based on the behavioral phenotype of the OT KO mice. In contrast, "learning and memory" ability for social recognition appears to be maintained in IT-related gene mutated medaka. Thus, studies using mutant medaka may allow us, for the first time, to dissect the neural/molecular mechanisms of complex information processes such as social motivation. Furthermore, the finding that social motivation toward unfamiliar individuals was recovered by learning after sexual maturity implies the presence of plastic neural networks that affect social motivation according to social familiarity in the adult medaka brain. By visualizing the neural activity of focal fish towards socially unfamiliar and familiarized fish by live imaging method and comparing them in the adult stage, we will be able to search for the plastic neural circuits that may regulate social motivation. Medaka fish is very small fish and transparent transgenic fish, in which the most pigment cells were genetically removed (Wakamatsu et al., 2001), is available. Therefore, medaka is considered to be good model organisms to perform imaging methods extensively. The analysis of neural networks in IT-related gene mutants will allow us to uncover the mechanism of social motivation according to social familiarity, which may be conserved from teleosts to mammals.

These findings in medaka are the first to unveil some of the molecular mechanisms underlying mate-guarding behavior in any species and provide a new avenue for studying the neural/molecular basis underlying the effects of social familiarity on social behaviors. My studies were focused mainly on the molecular mechanisms, and further analyses are needed to clarify the neural mechanisms underlying these social motivations. VP/VT and OT/MT/IT related genes are expressed broadly in the whole brain (Dubois-Dauphin et al., 1996; Huffman et al., 2012) and which neurons modulate social motivation is largely unknown in any species. Therefore, comprehensive genetic dissection of VT/IT system in medaka fish can uncover the whole mechanism underlying the regulation of social motivation, for which our current understanding is very poor.

Future prospects

Finally, I would like to describe my future plans for this research. In the present thesis, I showed that the medaka fish is an ideal animal for analyzing the mechanisms of social motivation, and the strategies described above will allow for the study of neural/molecular mechanisms of social motivation. After identifying some of the relevant neural circuits using medaka fish, I would like to examine whether the neural/molecular mechanisms of social motivation are conserved among vertebrates. Especially, I hope to investigate a possibility that similar mechanisms regulate human emotion or personality, such as jealousy and shyness. In humans, triadic relationships elicit jealousy, and motivation for mate-guarding could be assumed to be elicited by jealousy (Buss, 2002). Shy people do not tend to exhibit social behaviors toward unfamiliar people. In addition, the VP/OT systems also have important roles in social motivation in humans (Bartz et al., 2011). For example, male polymorphisms of the

5’ flanking region of V1aR (one of the VP receptors) gene is correlated with marital quality perceived by their partners, implying that VP modulates pair bonding in humans (Walum et al., 2008). Intranasal administration of OT specifically affects an individual's willingness to accept social risks arising from interpersonal interactions by increasing trust among humans (Kosfeld et al., 2005). The OT system is currently considered a promising candidate contributor of autism, because OT improves brain function in autistic children (Gordon et al., 2013). Taken together with these studies, I would like to propose a hypothesis that the mechanisms of the VP (VT)/OT (IT) system might be common between medaka and humans.

I expect that these studies will provide clues to elucidate the neural mechanisms underlying social motivation conserved among vertebrates and enhance our understanding of the evolutionary origin of jealousy and shyness in humans.

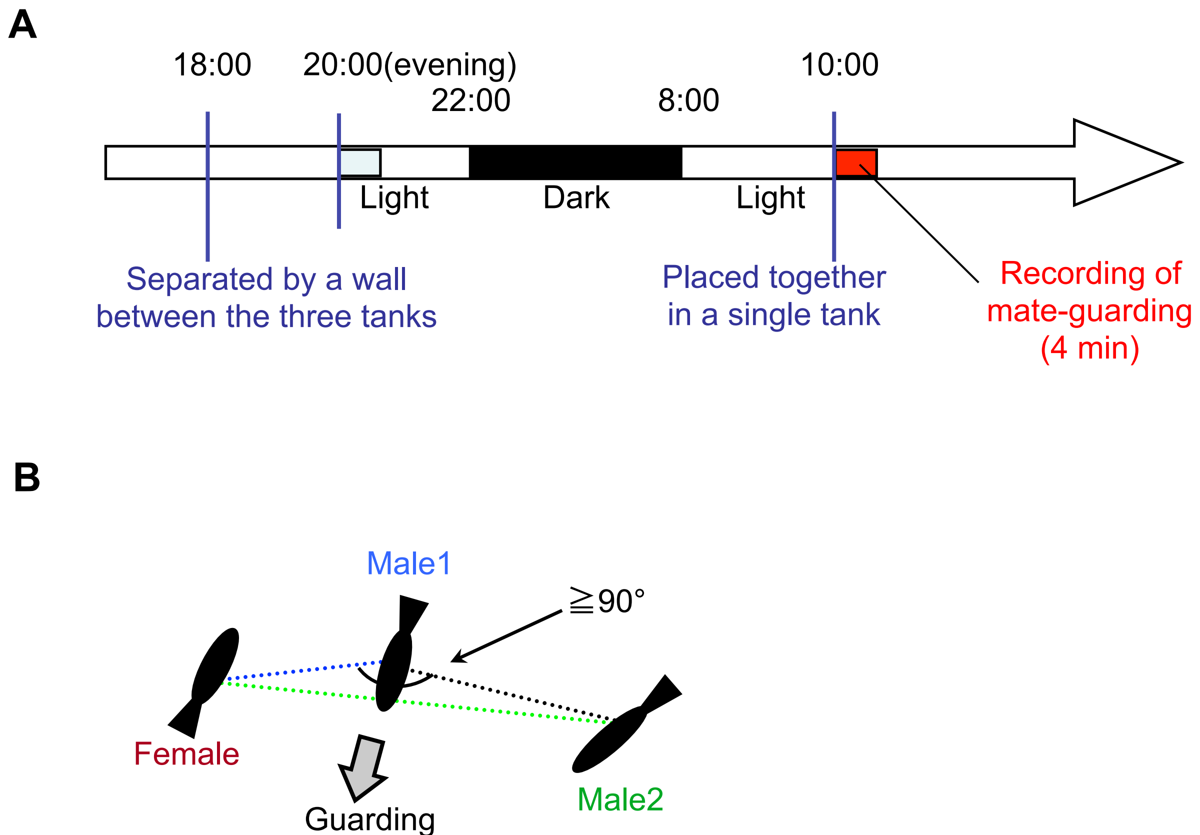
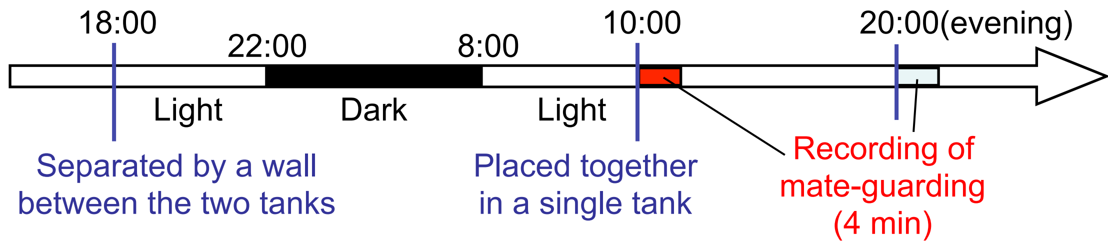
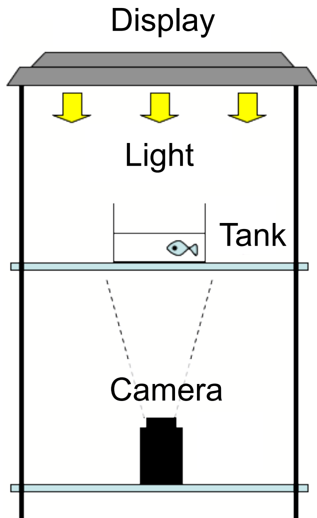
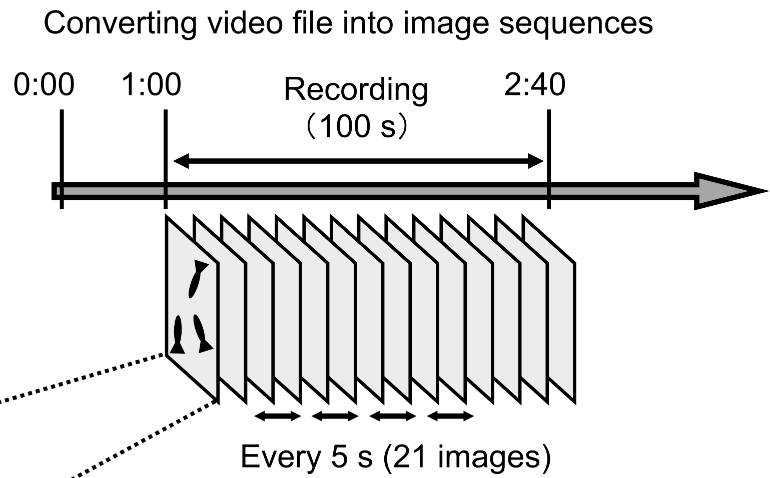
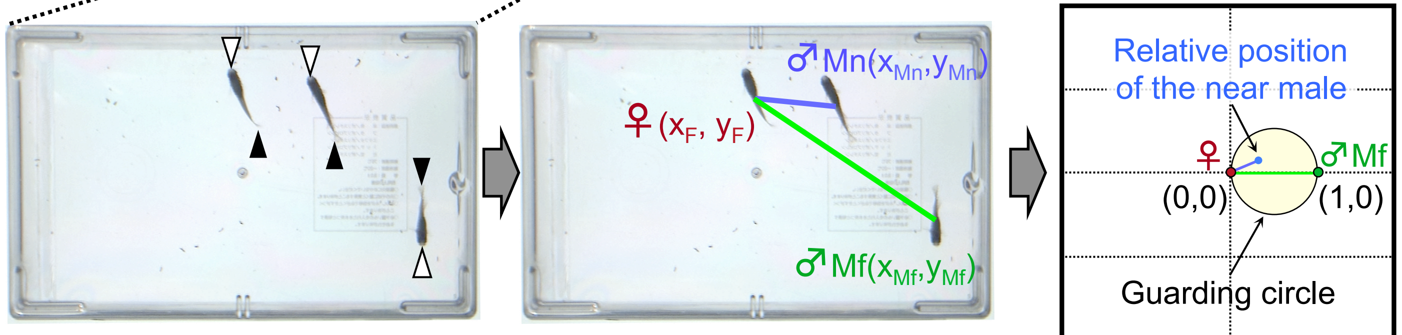


Figure 1 Observation of mate-guarding in a triangle relationship. (A) Time-course for the mate-guarding behavior observation. One female and two males were placed in an aquarium in the morning (~10:00) or in the evening (~20:00). Lights were turned off at 22:00. (B) One example of typical mate-guarding behavior. One male tended to maintain its position between the female and the other male and prevent that male from approaching the female. I defined this behavior as “mate-guarding behavior”.





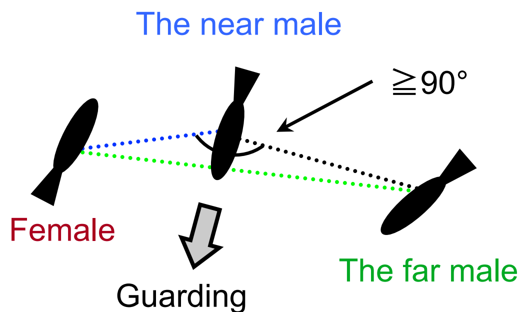


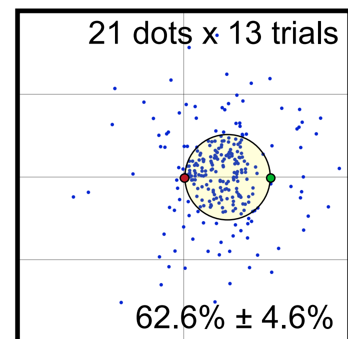


♂Mn …the near male

♂Mf …the far male

♀ …female





II

Guarding index of the near male

$$\text{Guarding index (\%)} = \frac{\text{The number of dots in the guarding circle}}{\text{21 dots}} \times 100$$

Figure 2 Procedure for the mate-guarding behavior assay. Upper left: Set-up for recording mate-guarding behavior. Fish movement was recorded from underneath, as the tank is transparent. Upper right: Time-course for the behavioral test. One female and two males were placed in an aquarium in the morning (~10:00) or in the evening (~20:00). Lights were turned off at 22:00. I converted video files into 21 image sequences per 5 s, and manually measured the head and tail positions of the three medaka fish using ImageJ (NIH) to calculate the center positions, which were used as their body positions. The male with a shorter mean distance for 100 s from the female than the other male was defined as the "near male", and the other male was defined as the "far male". Based on the positions of the female (x_F, y_F), the far male (x_{Mf}, y_{Mf}), and the near male (x_{Mn}, y_{Mn}), the relative positions of the near male (X, Y) were calculated by the formula described in the text when the positions of the female and the far male were defined as (0, 0) and (1, 0), respectively. I spotted the relative positions of the near male and defined the "guarding circle" as a circle with center (1/2, 0) and radius 1/2. When the near male is present in the guarding circle, the near male remains near the female and interferes with the rival (the far male). Thus, I defined the probability of being in the guarding circle as an index representing the degree of mate-guarding (guarding index).

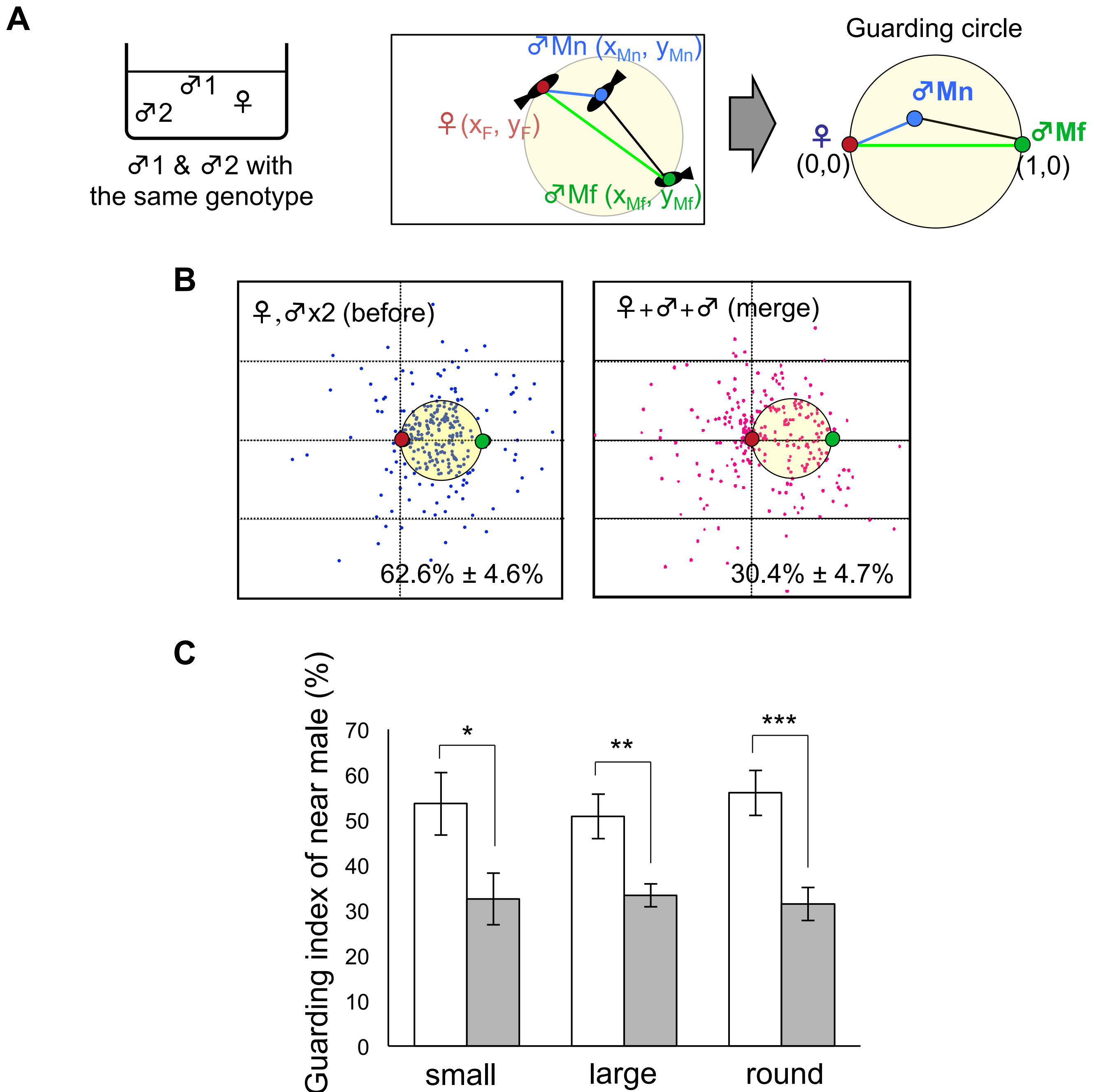
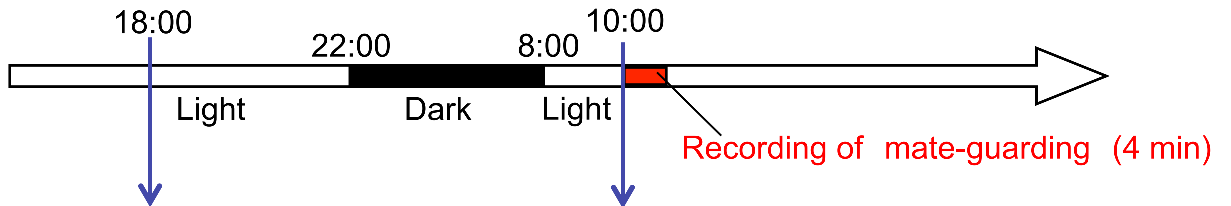


Figure 3 Guarding test to evaluate whether mate-guarding emerges. (A) A procedure for calculating the guarding index representing the degree of mate-guarding in the guarding test. As fish with the same genotype were used, I discriminated the two male fish according to the mean distance for 100 s from the female (near male and far male). I measured the relative locations of the three fish and calculated the probability of the near male being in the guarding circle with center (1/2, 0) and radius 1/2 when the female and far male positions were defined as (0, 0) and (1, 0), respectively. I defined this probability as the "guarding index of the near male" and compared the groups in the guarding test. (B) Relative positions of the three fish (one female and two males). Dots indicate the relative positions of the near male over 100 s (1 dot/5 s). Red and green small circles indicate positions of the female and far male, respectively. Guarding indices are shown in the lower right. "Merge" indicates superimposed data of three fish moving independently, and thus were used as a negative control. Each n = 21 dots x 13 trials. (C) Guarding indices using some different tanks. The size (small…9.5cm x 13 cm x 12 cm (height), large…21 cm x 30 cm x 10 cm (height)) and round shape (15 cm diameter circular tank) of the tank did not influence this behavior. Water depth was about 3-4 cm. Mean ± SEM. Each n = 12, Student's t-test: *P<0.05, **P<0.01, ***P<0.001.



A “before”

♂ ♂ ♀

♂ ♀ ♂

B “after”

♂ ♂ ♀

♂ ♂ ♀ ♂

mating

After removing ♂

♂ ♀ ♂

C “zebrafish female”

♂ ♂ ♀

Zebrafish ♀

♂ ♀ ♂

D “2 females + 1male”

♀ ♀ ♂

♀ ♂ ♀

E “3 males”

♂ ♂ ♂

♂ ♂ ♂

F “3 females”

♀ ♀ ♀

♀ ♀ ♀

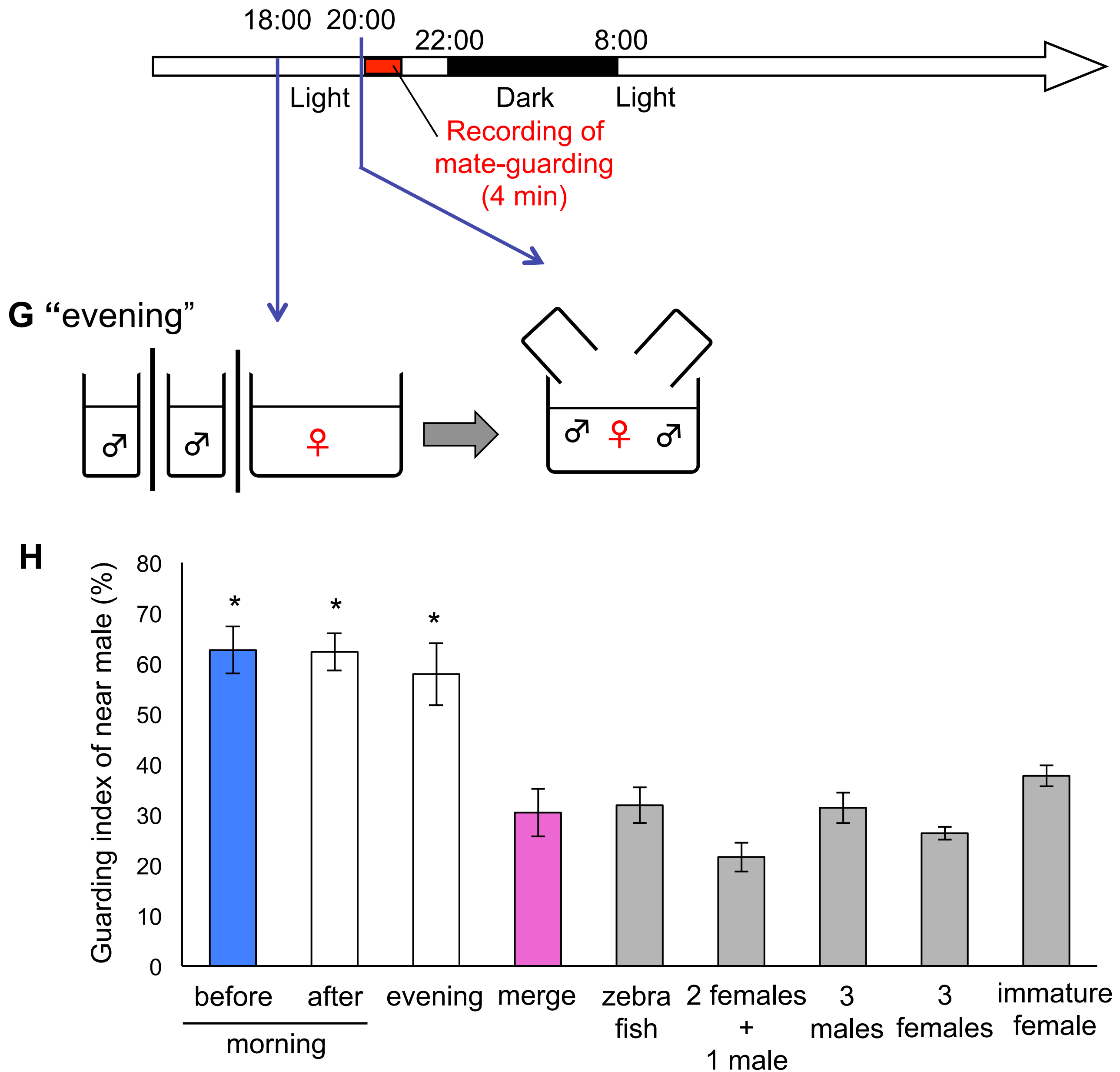


Figure 4 Time schedule and results of guarding tests in various conditions. (A) "Before": two males and a female were separated in the evening on the day before the assay and the next morning were placed together in a single tank. (B) "After": two males and a female were separated in the evening on the day before the assay. The next morning, I allowed a third male, which was not used in the guarding test to mate with the female. After that, I removed the male for mating and placed two males (separated on the day before the assay) and one female together in a single tank. (C) "Zebrafish female": two medaka males and a zebrafish female were separated in the evening on the day before the assay and the next morning, were placed together in a single tank. (D) "2 females + 1 male": two females and a male were separated in the evening on the day before the assay and the next morning were placed together in a single tank. (E) "3 males": three males were separated in the evening on the day before the assay and the next morning were placed together in a single tank. (F) "3 females": three females were separated in the evening on the day before the assay and the next morning were placed together in a single tank. (G) "Evening": two males and a female were separated in the evening and after ~2 hours were placed together in a single tank (20:00-21:00). (H) Comparison of guarding indices of the near male in the experimental group with that of the merged in guarding test. Mean ± SEM. Each n = 13, Dunnett's test **P*<0.05 VS "merge". Please see (A)-(G) for detail procedures for individual conditions.

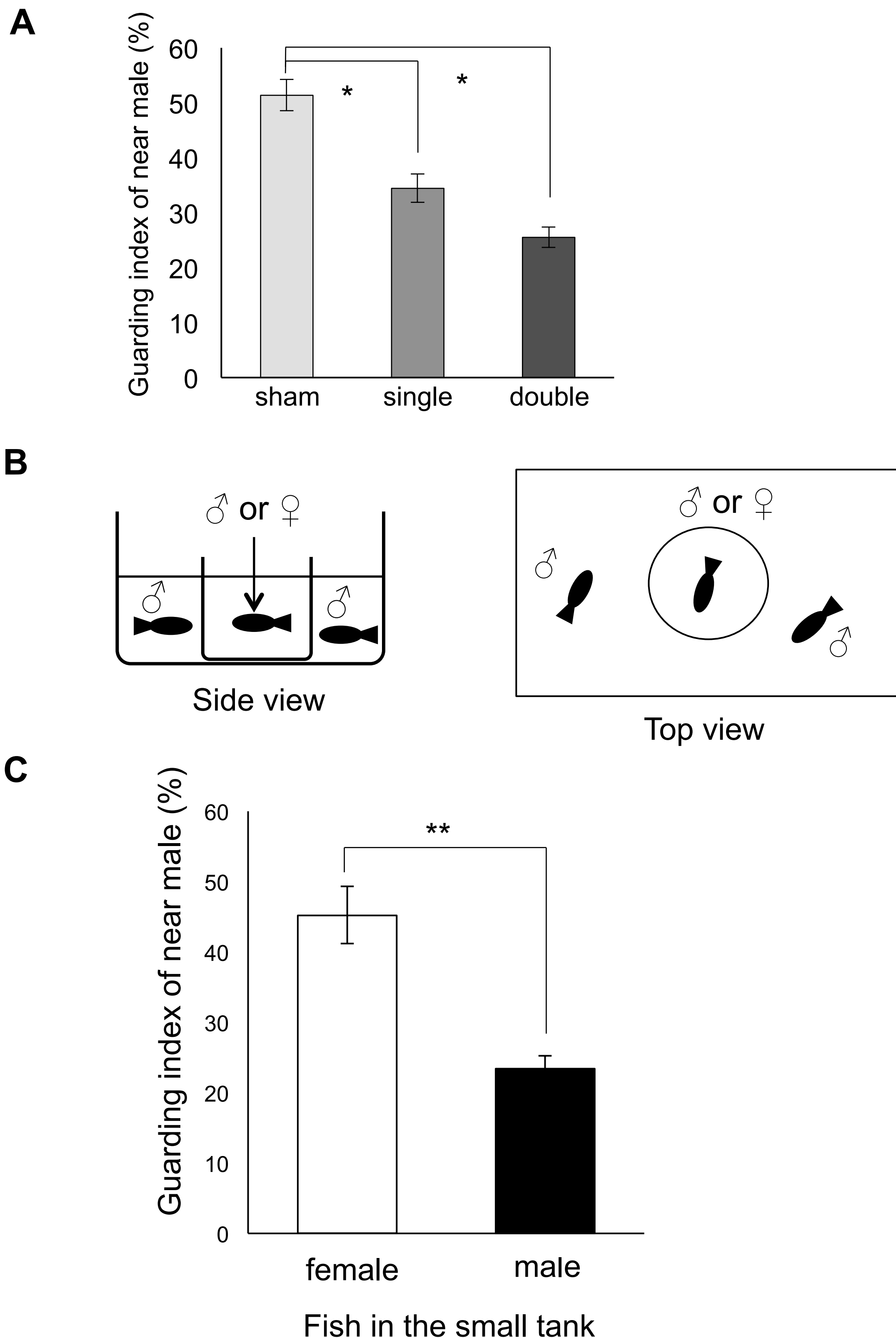


Figure 5 Requirement and sufficiency of visual information in mate-guarding behavior. (A) Unilateral and bilateral eye-ablated males did not exhibit mate-guarding. Sham fish were injured just above the eyes. Single and Double: One or two eyes were removed, respectively. Mean ± SEM. Each n = 12, Dunnett's-test: *P<0.05 VS "sham". (B) Procedure for the male or female isolated mate-guarding experiments, I placed a small transparent tank (6 cm diameter circular tank) in the center of the test tank. A female or male was placed in the small circular tank and two other males were placed in outside of the small tank (the water in the outer tank did not contain pheromones of the fish in the small tank). (C) Visual information is sufficient for males to exhibit mate-guarding. Mean ± SEM. Each n = 12, Student's t-test: **P<0.01.

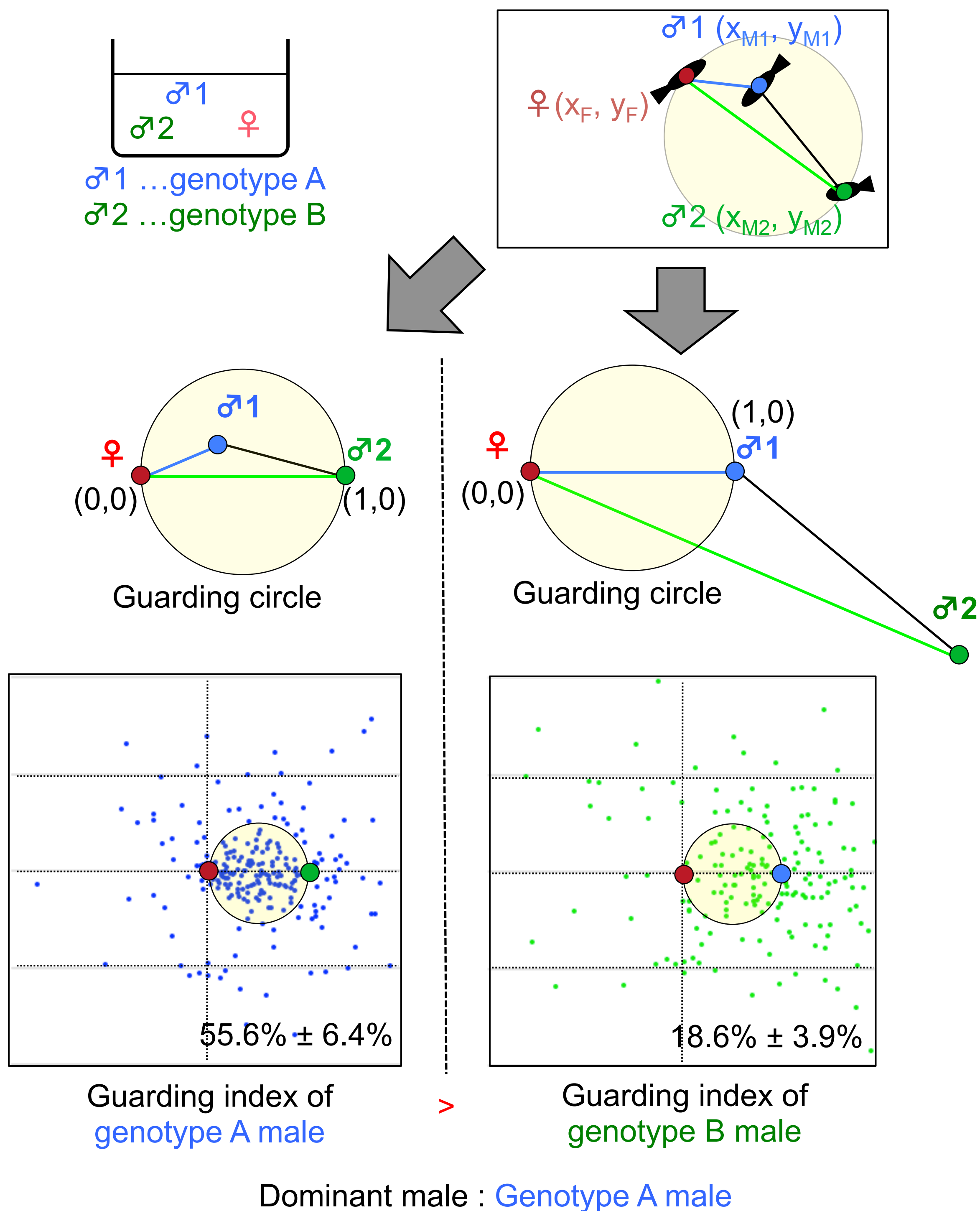


Figure 6 Comparison of the dominance in mate-guarding behavior (dominance test). I used one *genotype A* male and one *genotype B* male and measured their mate-guarding behavior when exposed to a female. I measured the relative locations of the three fish and calculated the probability of the *genotype A* male being in the guarding circle when the female and *genotype B* male positions were defined as (0, 0) and (1, 0), respectively (Left). I defined this probability as the "guarding index of *genotype A*". In contrast, I also calculated the probability of the *genotype B* male being in the guarding circle when the female and *genotype A* male positions were defined as (0, 0) and (1, 0), respectively (Right). I defined this probability as the "guarding index of *genotype B*" and compared with that of *genotype A*. A higher guarding index indicates higher dominance in the mate-guarding behavior compared with the other male.

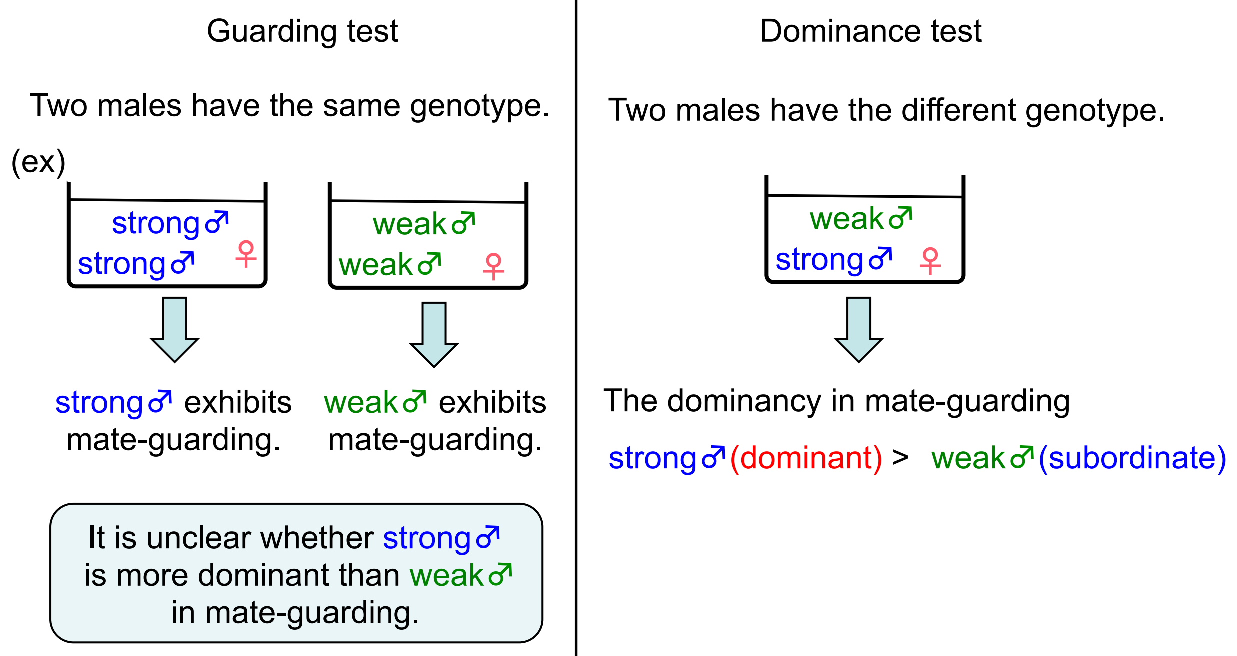


Figure 7 Difference between the guarding test and dominance test. In the guarding test, I used two males with the same genotype, so I could judge whether or not the males exhibited mate-guarding behavior. In this test, however, the mate-guarding can emerge irrespective of the strength of used males, as the guarding indices are altered according the strength of the rival males. In the dominance test, I can directly compare the guarding indices between two different genotypes, because a triadic setup comprises two males with different genotypes.

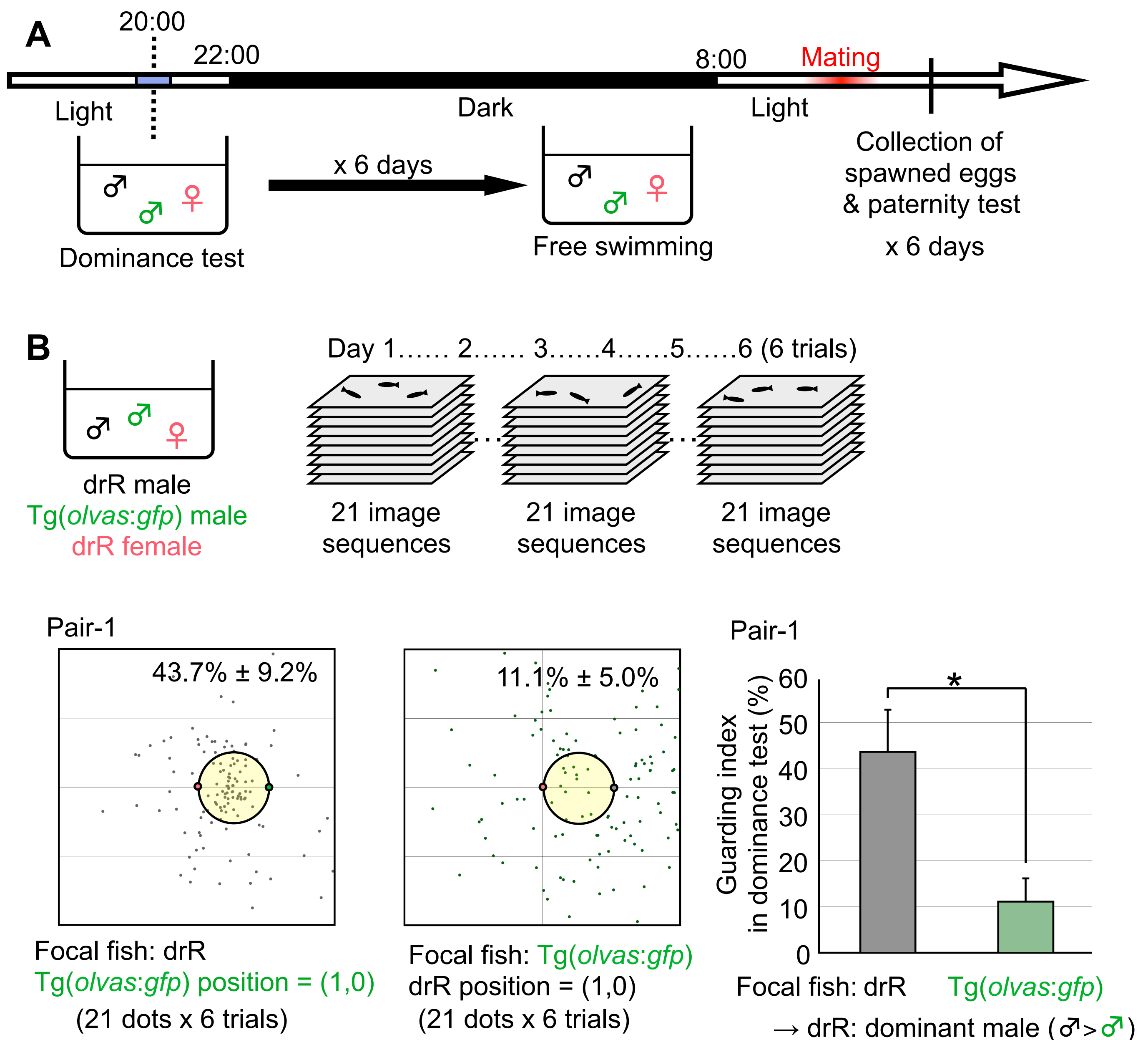


Figure 8 Procedure for the dominance and paternity tests. (A) Time-course for the dominance and paternity tests. (B) I integrated the results of a 6-d dominance test and compared the guarding index of each genotype and judged which male was dominant by Mann-Whitney U test. Pair-1 is shown as an example. The guarding index of the wild-type (drR) male (43.7%) was significantly higher than that of the transgenic (Tg; *homozygote olvas*:*gfp*) male (11.1%; Mann-Whitney U test, P=0.020, n = 6). In this case, I judged that the wild-type (drR) male was dominant. If there was no significant difference (Mann-Whitney U test, P>0.05, n = 6), I judged that the two fish were equal.

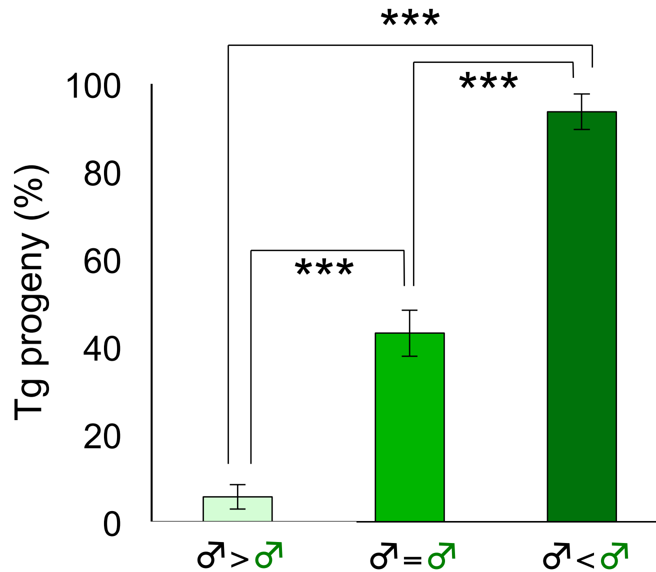


Figure 9 Higher mating success rate of the dominant male. 17 pairs were classified into three groups: the wild-type dominant pairs (n = 7, ♂ > ♂), Tg(*homozygote olvas*:*gfp*)-dominant pairs (n = 5, ♂ < ♂), and equivalent pairs (n = 5, ♂ = ♂). I compared the percentage of GFP-positive eggs, indicating the Tg progeny rate, among the three groups. Mean ± SEM. n's = 7, 5, 5, respectively. one-way ANOVA: Scheffé's post-hoc ***$P<0.001$.

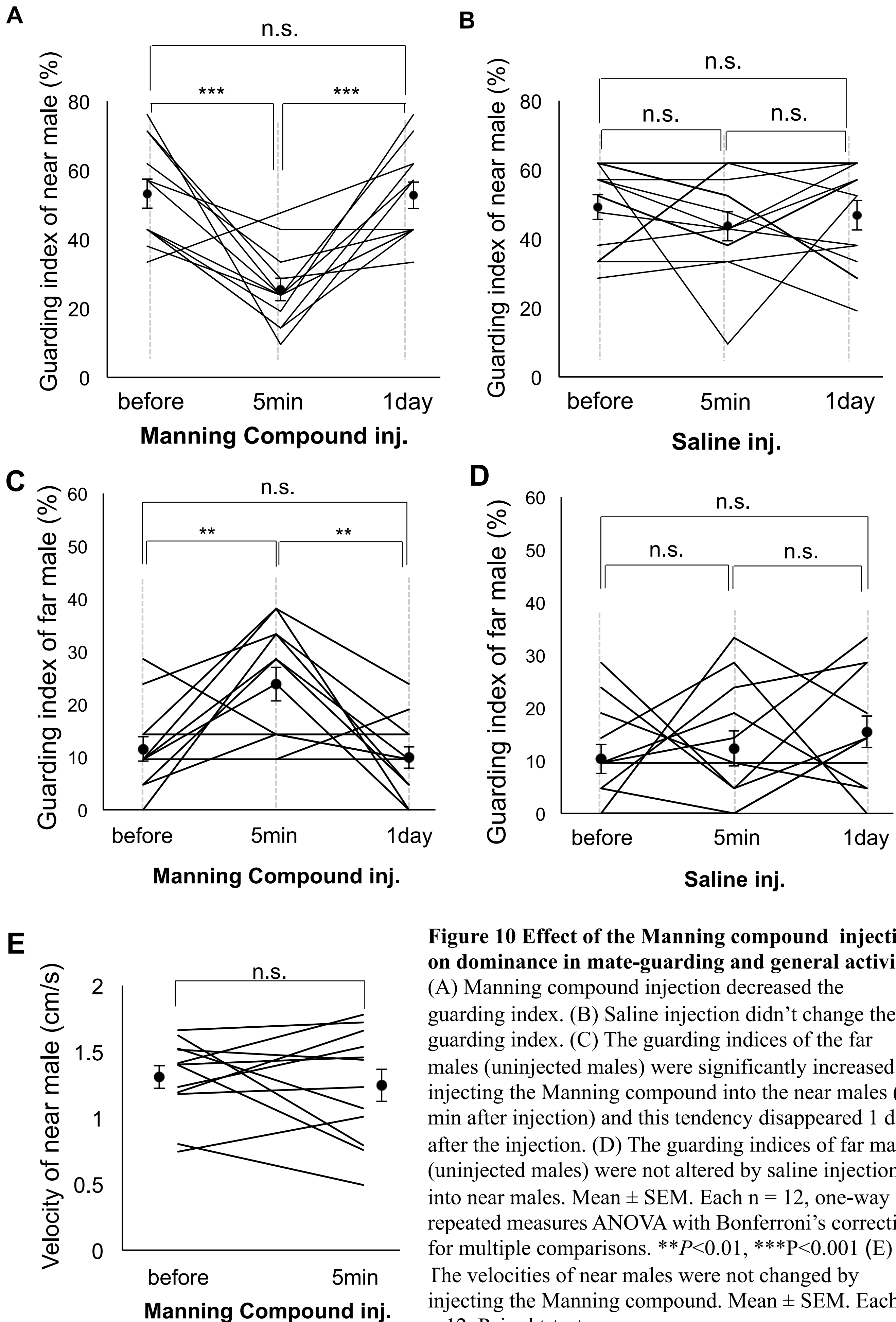


Figure 10 Effect of the Manning compound injection on dominance in mate-guarding and general activity. (A) Manning compound injection decreased the guarding index. (B) Saline injection didn't change the guarding index. (C) The guarding indices of the far males (uninjected males) were significantly increased by injecting the Manning compound into the near males (5 min after injection) and this tendency disappeared 1 day after the injection. (D) The guarding indices of far males (uninjected males) were not altered by saline injection into near males. Mean ± SEM. Each n = 12, one-way repeated measures ANOVA with Bonferroni's correction for multiple comparisons. $^{**}P<0.01$, $^{***}P<0.001$ (E) The velocities of near males were not changed by injecting the Manning compound. Mean ± SEM. Each n = 12, Paired t-test.

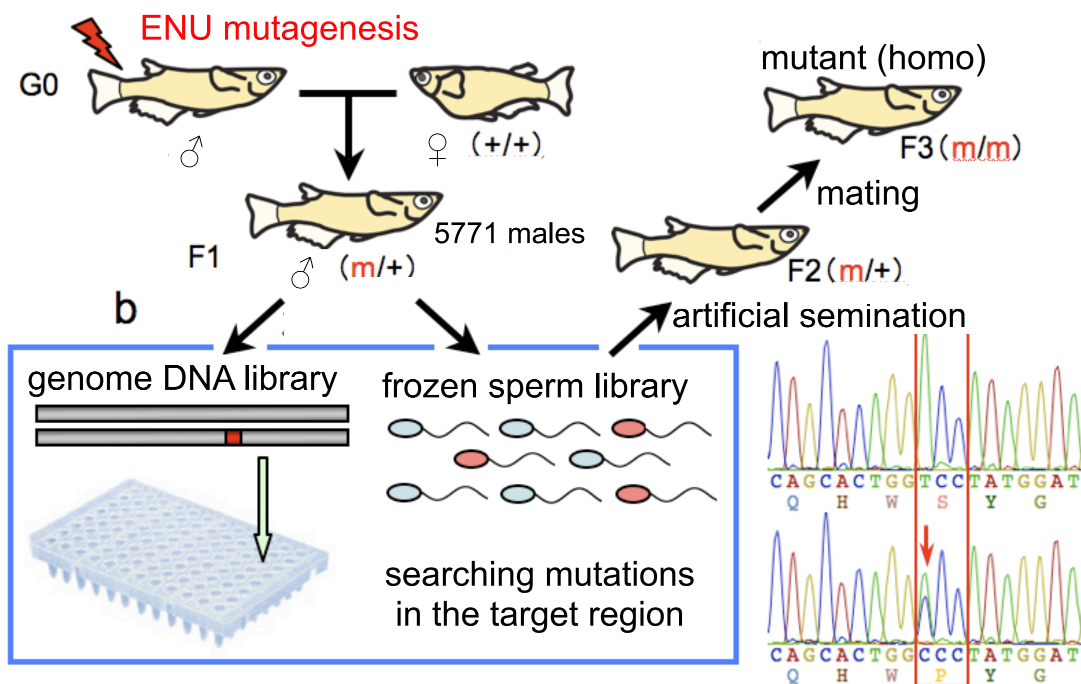
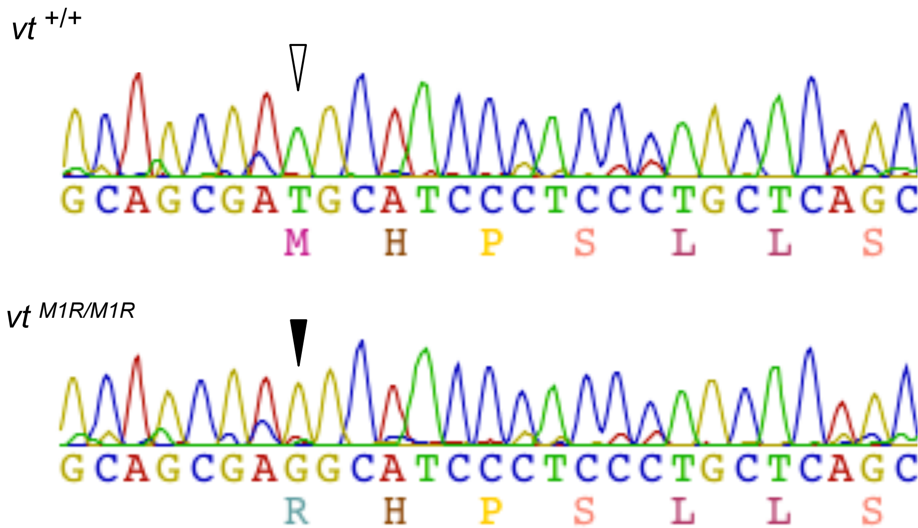


Figure 11 Procedure for TILLING methods. TILLING library is composed of sets of frozen sperm and genomic DNA which were prepared from 5771 F1 males generated by crossing wild-type females and N-nitroso-N-ethylurea (ENU)-mutagenized males (Taniguchi et al., 2006). I amplified target regions of each genes using PCR, and identified mutations in PCR products by HRM (high-resolution melting curve) analysis and sequencing. Artificial semination from frozen sperm library allows us to get target gene mutated medaka.

A



B

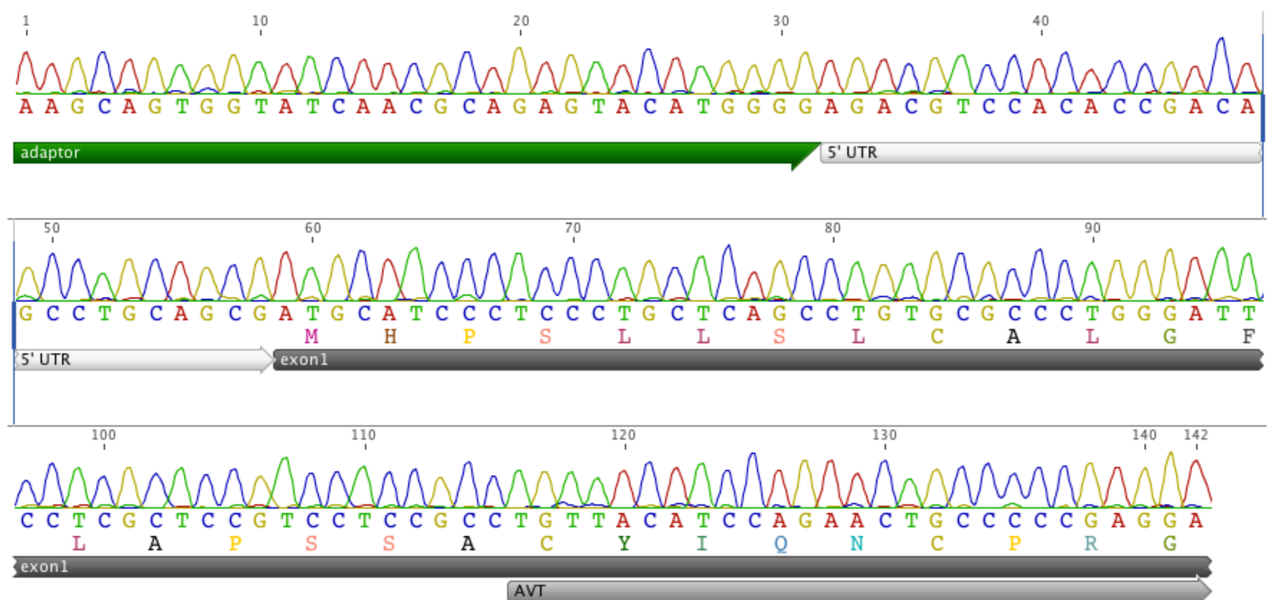


Figure 12 Screening for *vt* mutant alleles by TILLING. (A) A local sequence dataset comparing the wild-type *vt* ($vt^{+/+}$) and vt^{M1R} homozygotes ($vt^{M1R/M1R}$) demonstrating the *vt* T2G mutation in vt^{M1R} mutants (black arrowhead). (B) A local sequence of 5'-RACE product confirming the transcription initiation site of *vt*, which was predicted by the annotated *vt* sequence. I sequenced 10 and 9 cDNA clones derived from the drR and cab strains, respectively and confirmed that the sequences of all 19 cDNA clones started from the transcription initiation site, which was predicted by the annotated *vt* sequence. "adaptor" : added nucleotide in SMARTer RACE cDNA Amplification Kit (Clontech).

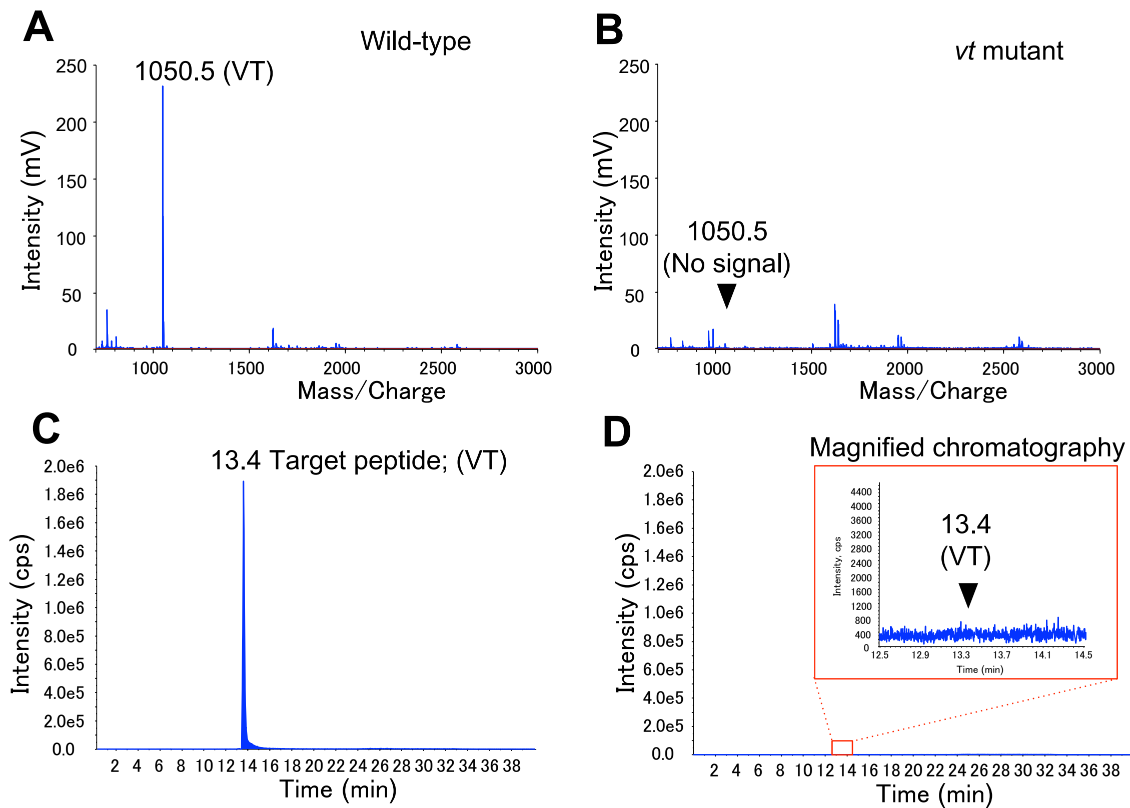
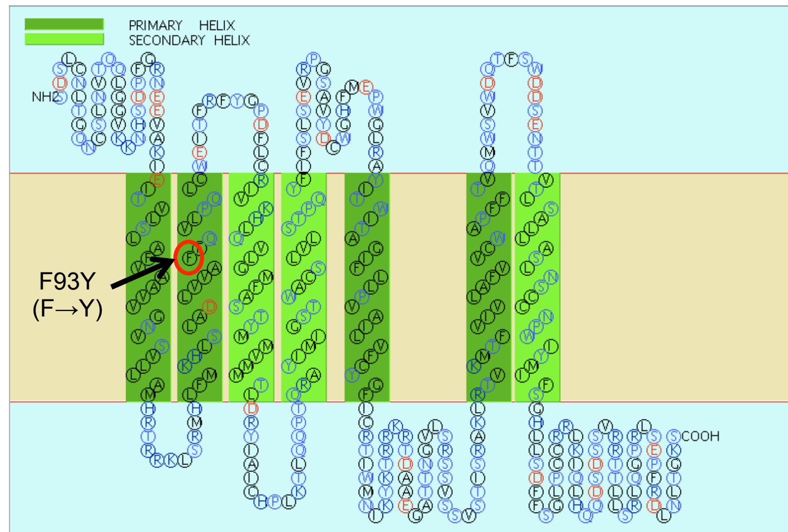


Figure 13 Mass spectrometry analysis (Peptide MALDI-TOF MS analysis and SRM assay). VT peptides are present in the pituitary of the wild-type (Cab), but not in the *vt* mutant. MALDI-TOF MS spectra of the peptides from the pituitary in wild-type (A) and *vt* mutant (B). The x-axis shows the m/z, mass to charge ratio; the y-axis shows the intensity of the molecular ions. An ion peak at m/z 1050.5 indicated the presence of the VT peptide in the wild-type (A). For the SRM assay, I selected Q1 (precursor ion: 525.8)/Q3 (fragment ion y3: 328.2), based on tandem MS spectrum of [Arg8]-vasotocin (Sigma Aldrich, V0130). Abundant VT peptides were detected in the wild-type (C), while no VT peptide was detected in *vt* mutant (D). The x-axis shows retention time; the y-axis shows the intensity of the molecular ions.



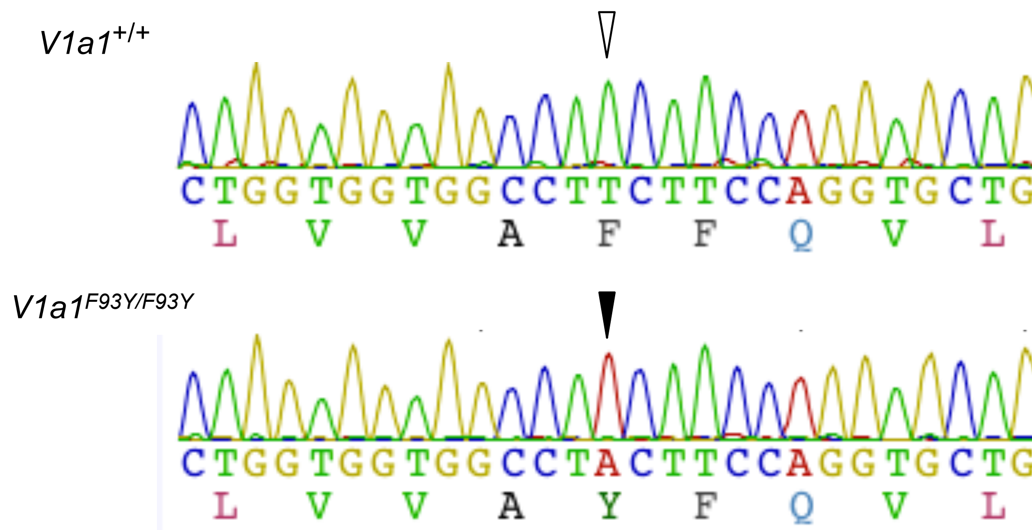
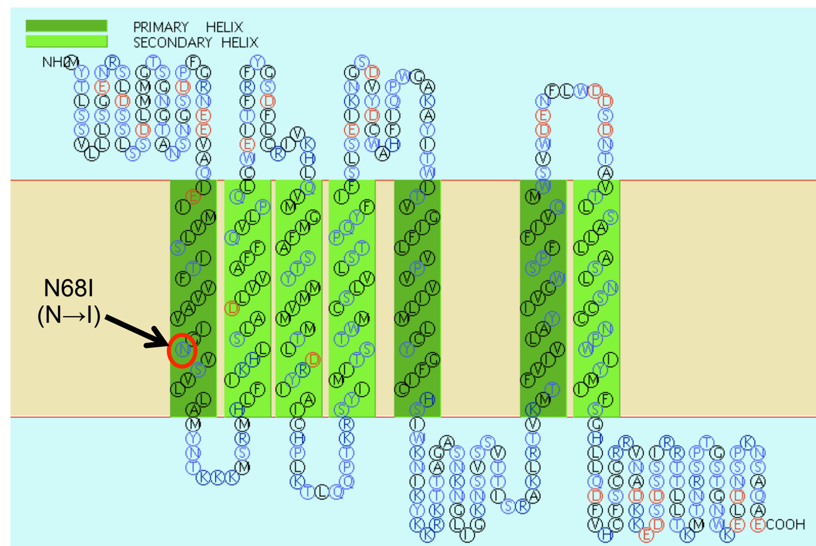


Figure 14 Screening for *V1a1* mutant alleles by TILLING. A local sequence dataset comparing the wild-type *V1a1* ($V1a1^{+/+}$) and $V1a1^{F93Y}$ homozygotes ($V1a1^{F93Y/F93Y}$) demonstrating the *V1a1* T278A mutation in $V1a1^{F93Y}$ mutants (black arrowhead).



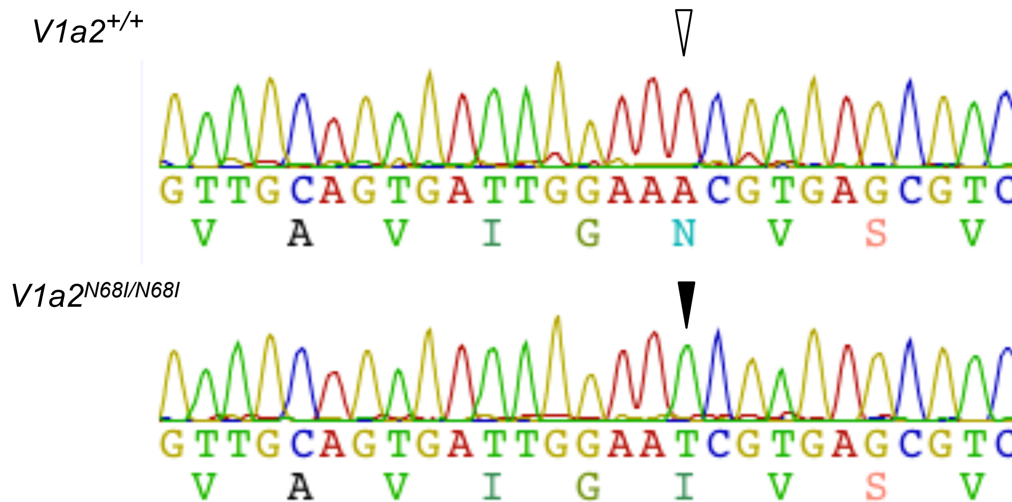


Figure 15 Screening for *V1a2* mutant alleles by TILLING. A local sequence dataset comparing the wild-type *V1a2* ($V1a2^{+/+}$) and $V1a2^{N68I}$ homozygotes ($V1a2^{N68I/N68I}$) demonstrating the *V1a2* A203T mutation in $V1a2^{N68I}$ mutants (black arrowhead).

species	gene	Amino acid sequence														
mouse	*V1aR*	G	N	S	S	V	L	L	…	V	A	F	F	Q	V	L
fugu	*V1a1*	G	N	V	S	V	L	L	…	V	A	F	F	Q	V	L
fugu	*V1a2*	G	N	V	S	V	L	L	…	V	A	F	F	Q	V	L
medaka	*V1a1*	G	N	V	S	V	L	L	…	V	A	F	F	Q	V	L
medaka	*V1a2*	G	N	V	S	V	L	L	…	V	A	F	F	Q	V	L
medaka	*V1a1^{F93Y}*	G	N	V	S	V	L	L	…	V	A	Y	F	Q	V	L
medaka	*V1a2^{N68I}*	G	I	V	S	V	L	L	…	V	A	F	F	Q	V	L

Figure 16 The primary structure of V1a receptor paralogs. The primary structure of V1a receptor paralogs in mouse and fish. Phenylalanine 93 and arginine 68, which are identical among known forms, were changed to tyrosine and isoleucine in V1a1^{F93Y} and V1a2^{N68I} mutant alleles, respectively (red letters).

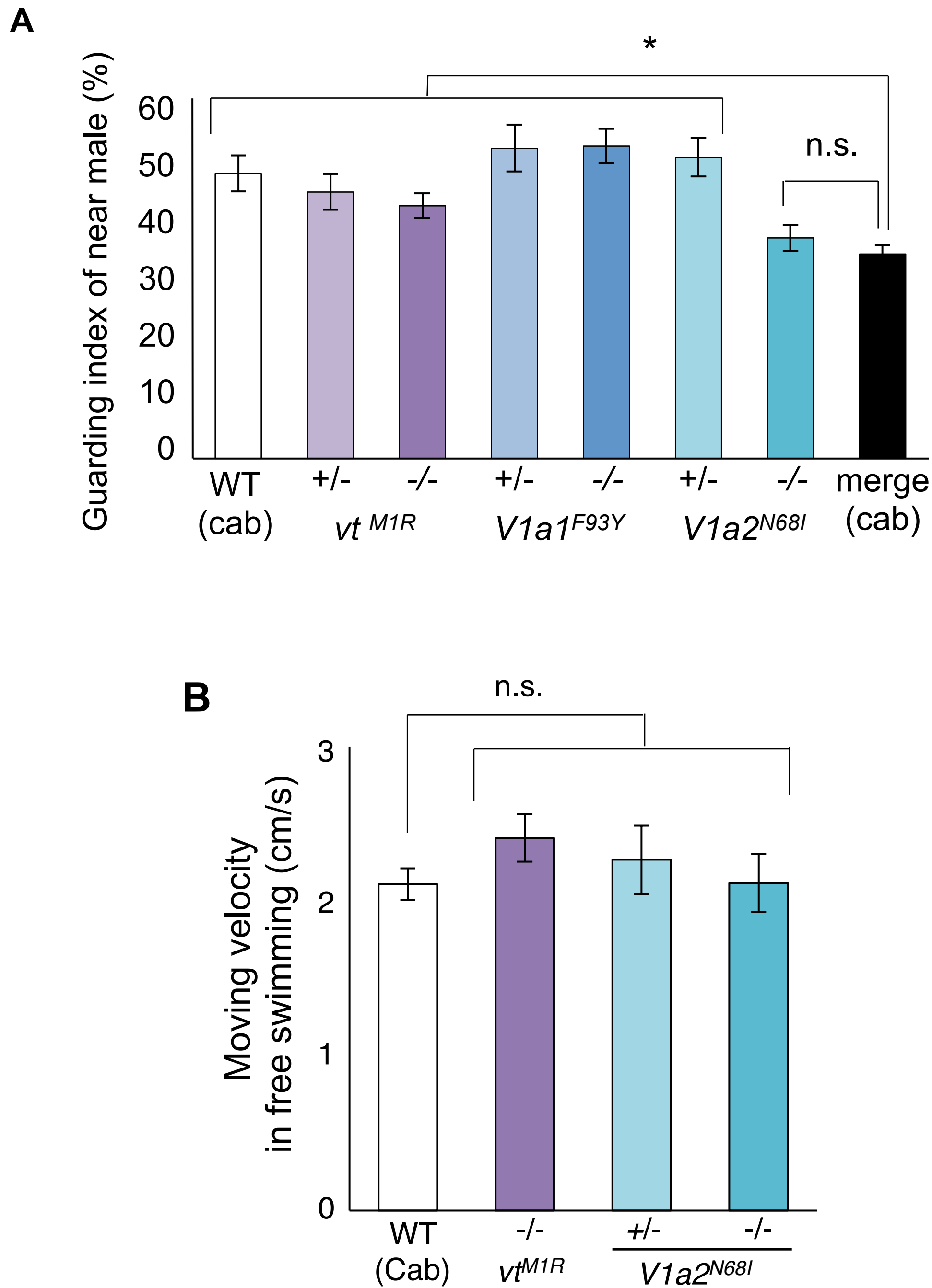
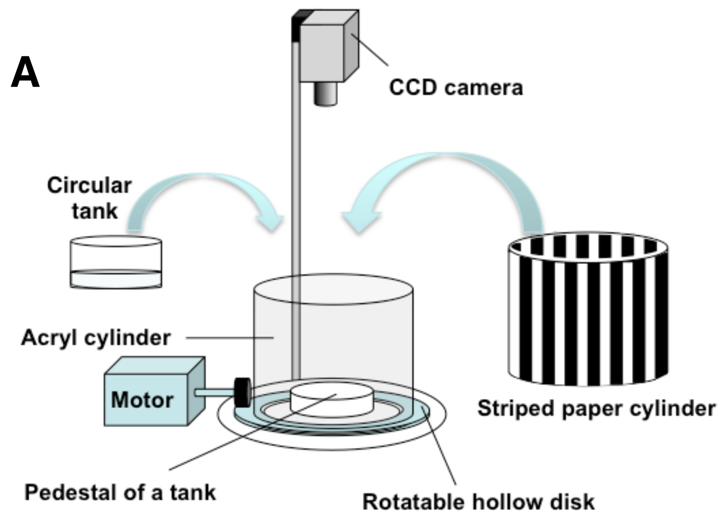
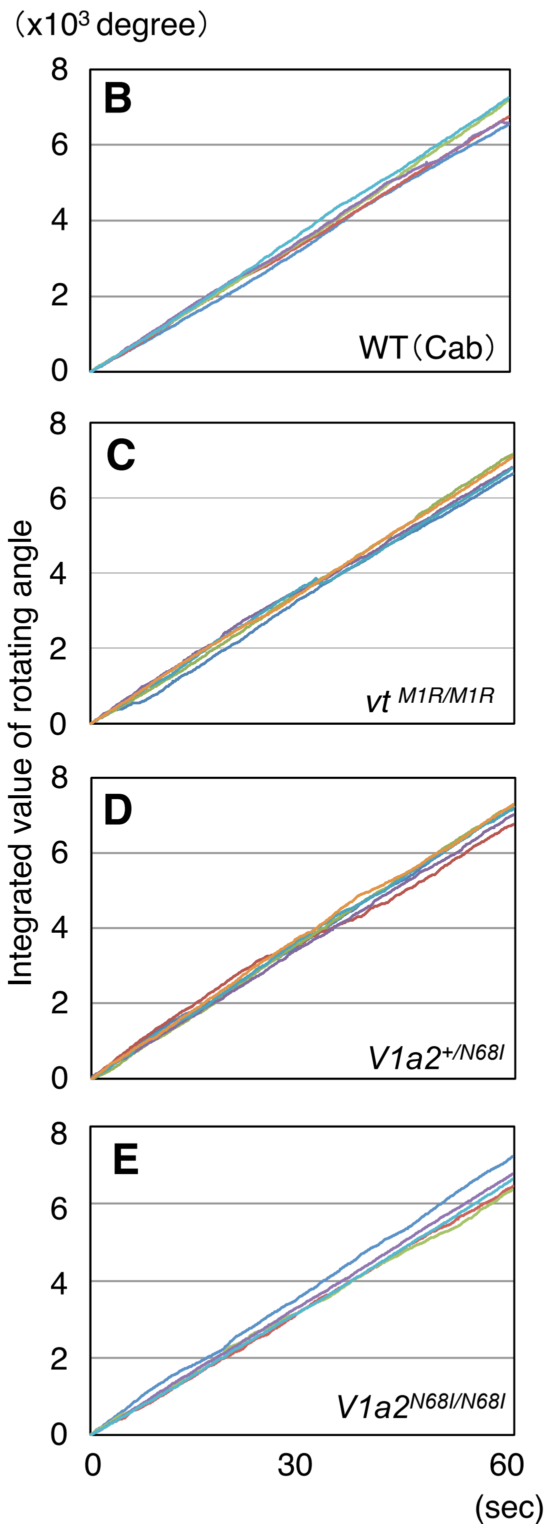


Figure 17 The guarding test using VT and VT receptors gene mutants and the velocity in free swimming of those mutants . (A) Effect of the VT system on emergence of mate-guarding behavior (guarding test). *vt* ($vt^{M1R/M1R}$) and *V1a1* ($V1a1^{F93Y/F93Y}$) mutants exhibited mate-guarding behavior, whereas *V1a2* ($V1a2^{N68I/N68I}$) mutants did not. Mean ± SEM. Each n = 12, Dunnett's test : *P<0.05. (B) Normal free swimming velocity in $vt^{M1R/M1R}$, $V1a2^{+/N68I}$, and $V1a2^{N68I/N68I}$ male fish. One minute after I placed 1 fish in the tank, I calculated its movement velocity for 60 s. Mean ± SEM. Each n = 4, Dunnett's test.





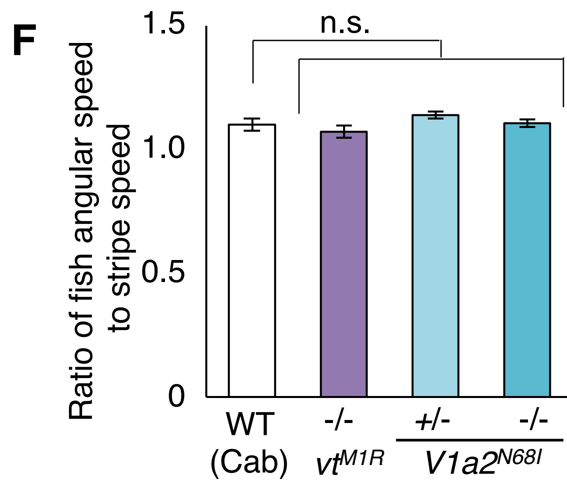
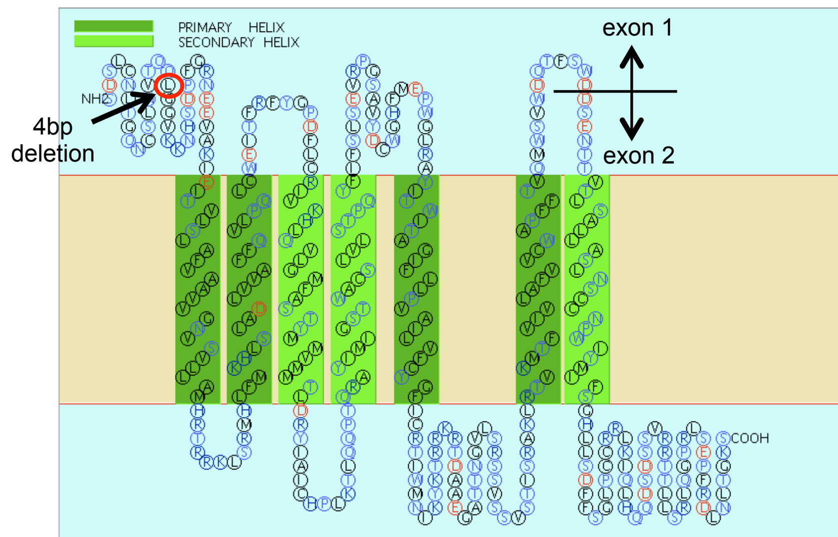
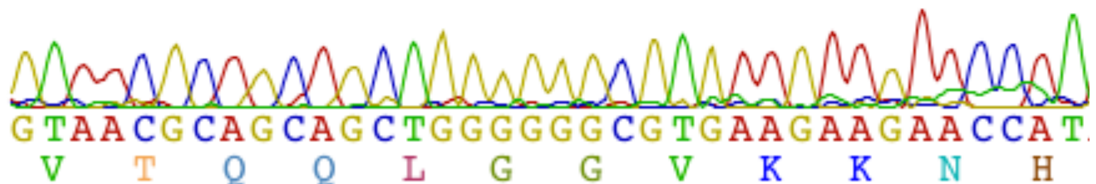


Figure 18 Normal optomotor response (OMR) in ***vt***$^{M1R/M1R}$**,** ***V1a2***$^{+/N68I}$ **and** ***V1a2***$^{N68I/N68I}$ **male fish.**
(A) Equipment for the analysis of the OMR described previously (Imada et al., 2000). (B-E) Integrated angular velocity during 60 s of (B) wild-type (Cab), (C) *vt*$^{M1R/M1R}$, (D) *V1a2*$^{+/N68I}$, and (E) *V1a2*$^{N68I/N68I}$ fish. Each line indicates integrated angular velocity of five individual fish. (F) Ratio of the mean fish angular speed to that of the stripe speed. Mean ± SEM. Each n = 5, Dunnett's test.



V1a1$^{+/+}$



V1a1 KO (4bp deletion)

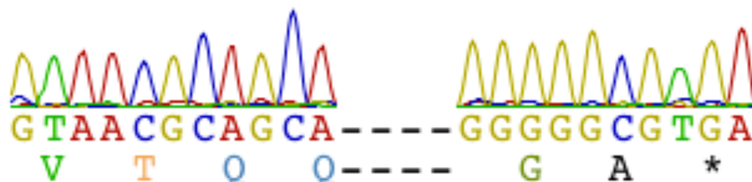
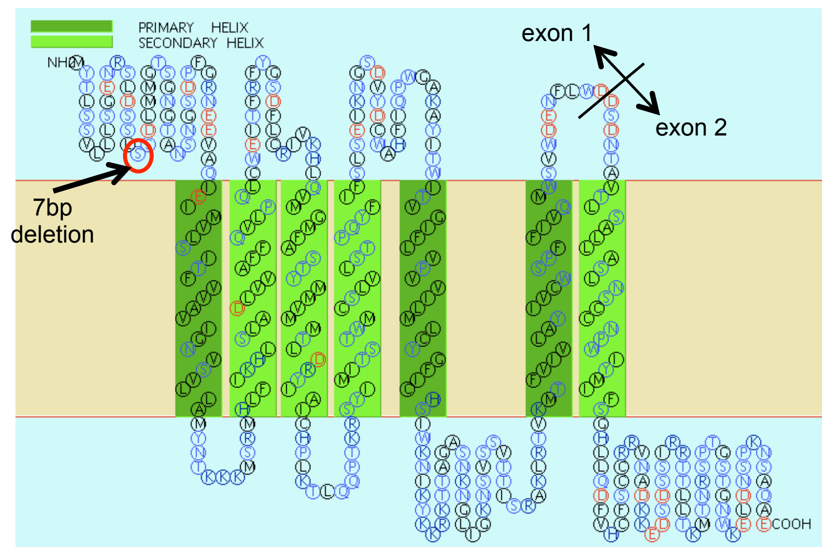
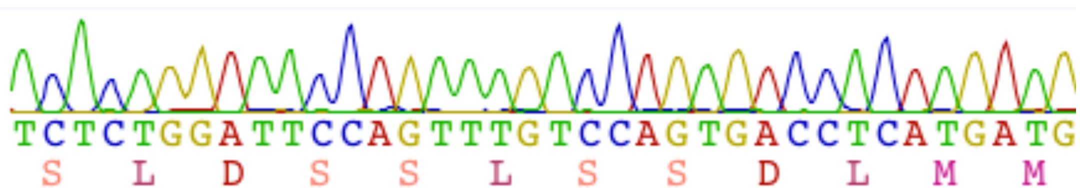


Figure 19 Generation of *V1a1* mutant alleles by TALEN. A local sequence dataset comparing wild-type *V1a1* (*V1a1*$^{+/+}$) and V1a1 knockout (KO) homozygotes demonstrating that a 4-bp deletion generated a nonsense mutation (G26X) in *V1a1* KO mutants. *V1a1* gene consists of two exons. The deletion was located in the first exon and the mutated transcripts encode C-terminal deleted proteins lacked six of the seven transmembrane domains encoded by the first exon.

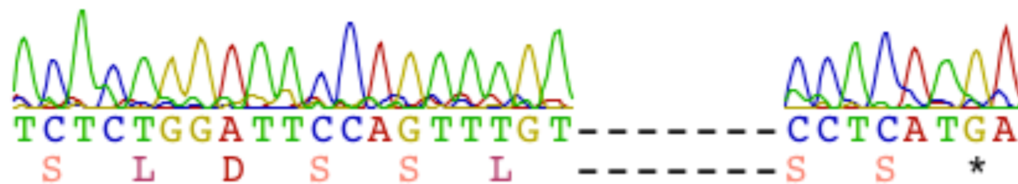
A



V1a2$^{+/+}$



V1a2 KO (7bp deletion)



B

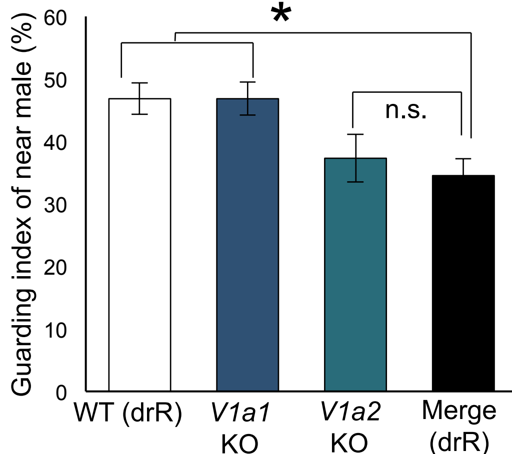


Figure 20 Generation of *V1a2* mutant alleles by TALEN and mate-guarding behavior of *V1a1* and *V1a2* knockout mutants. (A) A local sequence dataset comparing the wild-type *V1a2* (*V1a2*$^{+/+}$) and *V1a2* knockout (KO) homozygotes demonstrating that a 7-bp deletion generated a nonsense mutation (D24X) in *V1a2* KO mutants. *V1a2* gene consists of two exons. The deletion was located in the first exon and the mutated transcripts encode C-terminal deleted proteins lacked six of the seven transmembrane domains encoded by the first exon. (B) *V1a1* KO mutants exhibited mate-guarding behavior, whereas *V1a2* KO mutants did not. Mean ± SEM. Each n = 12, Dunnett's test: *$P<0.05$ VS "merge".

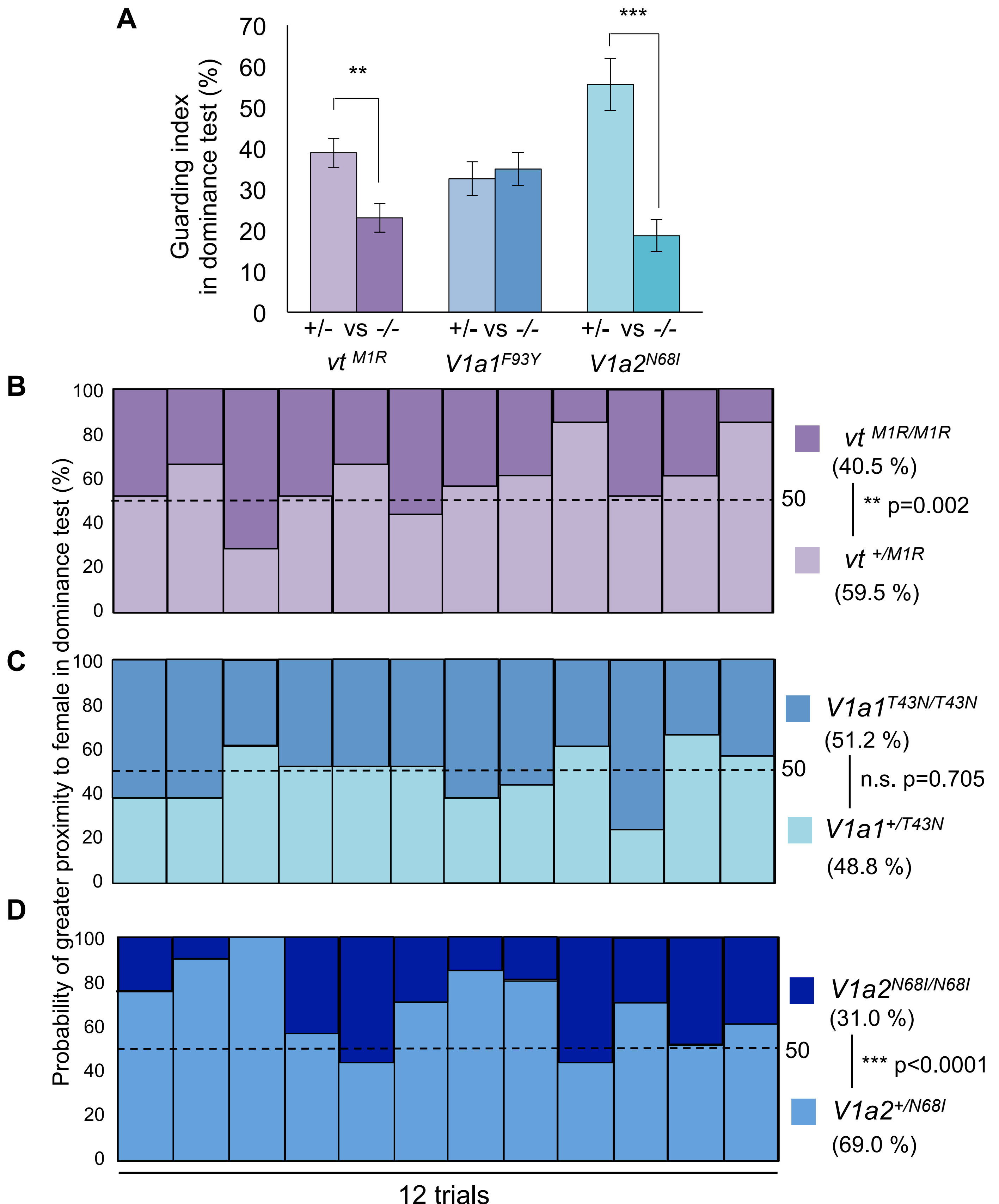


Figure 21 Effect of the VT system on dominance in mate-guarding behavior (dominance test). (A) *vt* homozygote mutants ($vt^{M1R/M1R}$) and *V1a2* homozygote mutants ($V1a2^{N68I/N68I}$) tended to be subordinate males in the dominance test. Mean ± SEM. Each n = 12, Student's t-test: **P<0.01, ****P*<0.001. (B-D) Dominant males in the mate-guarding behavior maintained closer proximity to the female than subordinate males. I judged which male was closer to the female in a total 256 images (21 images x 12 trials) in the dominance test. Here I calculated the probabilities of being closer to the female between heterozygote and homozygote mutants based on the 256 images. I then detected a significant bias between the two probabilities using the chi-square test in *vt* and *V1a2* mutants, but not in *V1a1* mutants.

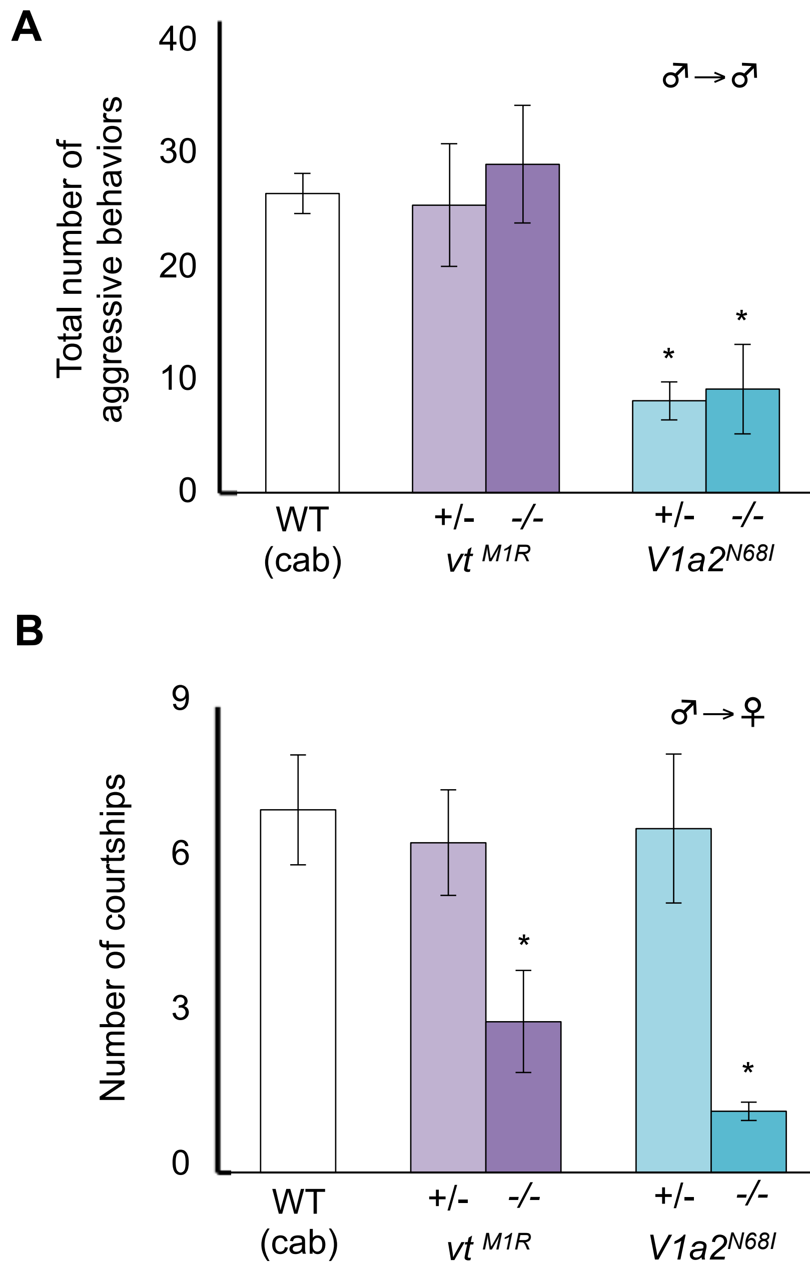


Figure 22 Effect of VT-related genes mutations on aggressive behavior (intrasexual interaction) and courtship behavior (intersexual interaction). (A) *vt* mutant males exhibited normal aggression, whereas *V1a2* heterozygote and homozygote mutant males exhibited low aggression. Mean ± SEM. Each n = 8, Dunnett's test: *P<0.05 VS wild-type. (B) *vt* and *V1a2* mutant males showed lower motivation to mate than wild-type males. Mean ± SEM. Each n = 11, Dunnett's test: *P<0.05 VS wild-type.

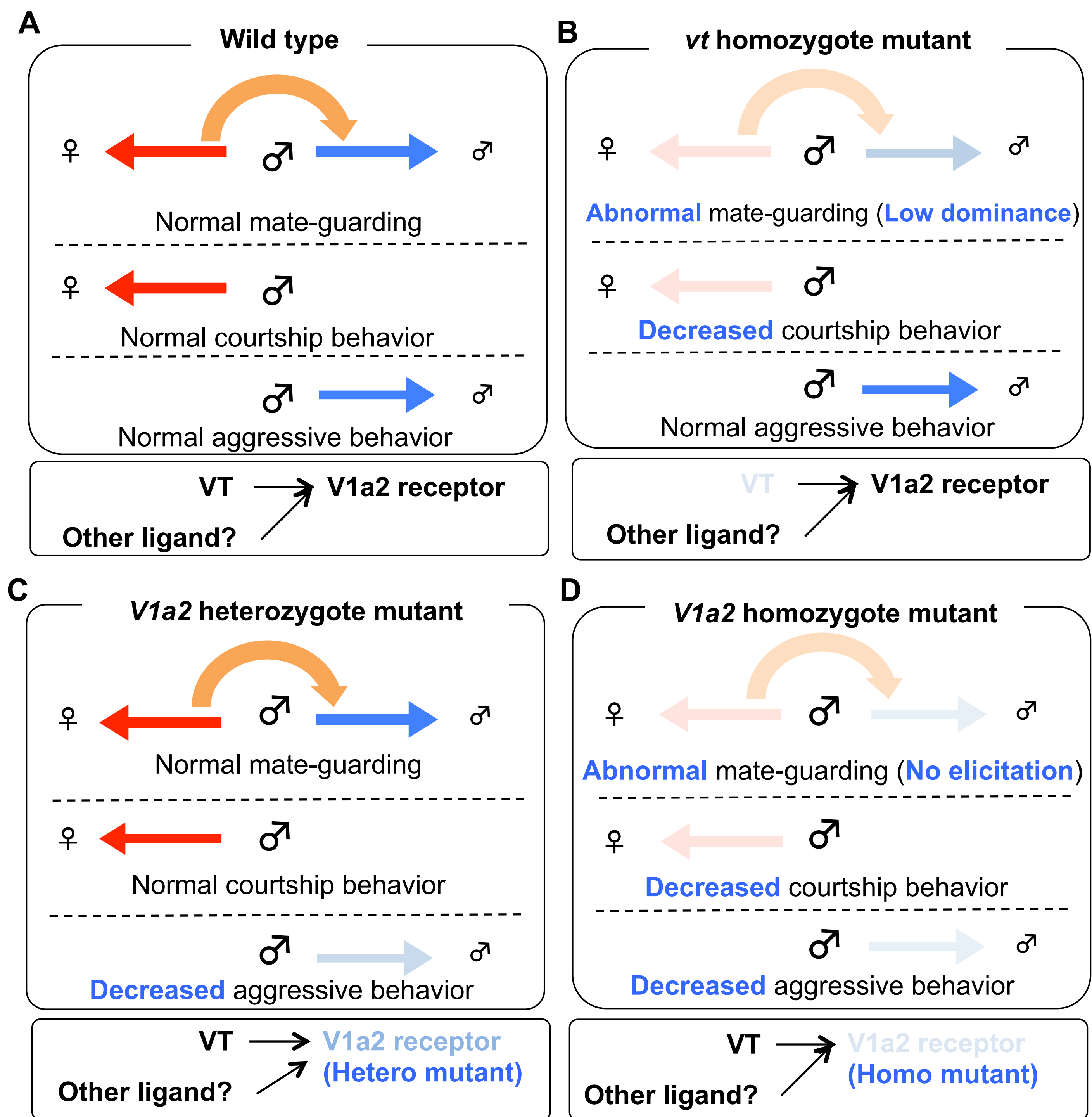
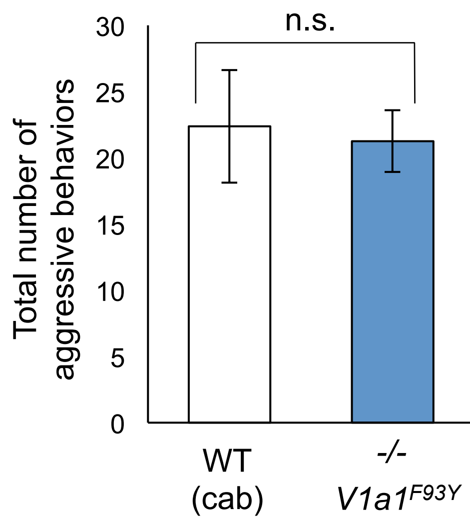


Figure 23 Summary of behavioral phenotype and related VT system. (A) Mate-guarding requires two different types of motivation: sexual motivation toward the opposite sex and competitive motivation toward the same sex, while courtship and aggressive behaviors require social motivation to either the opposite (red arrow) or same (blue arrow) sexes. When mate-guarding emerges in a triadic relationship, the presence of a potential mating partner may engage competitive motivation toward rival males via VT system, leading to male-male competition (orange arrow). (B) The homozygote *vt* mutant males normally have sexual motivation, but not competitive motivation toward the same sex. Decreased sexual motivation may cause low dominance of male-male competition in mate-guarding. *vt* was not required for either normal aggressive behaviors or elicitation of mate-guarding, suggesting the presence of redundant system activating V1a2 receptor. (C) The heterozygote *V1a2* mutants normally have competitive motivation, but not sexual motivation toward the opposite sex. Decreased competitive motivation has no effect on male-male competition in mate-guarding, because sexual motivation may dominantly drive the motivation for mate-guarding. The single functional *V1a2* allele may not produce enough of a gene product, leading to attenuated aggression. (D) The homozygote *V1a2* mutants did not normally have social motivation to either the same sex or opposite sex. *V1a2* was required for elicitation of mate-guarding.



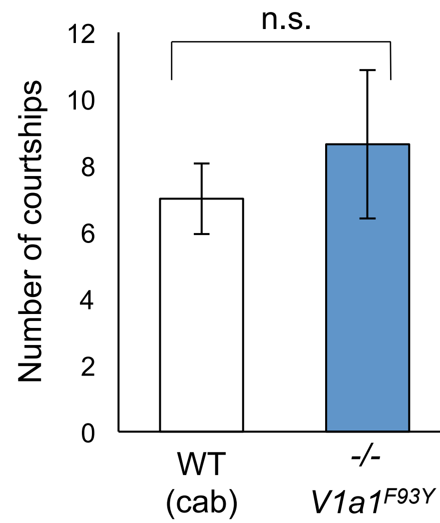


Figure 24 Lack of effect of *V1a1* mutation on aggressive behavior or courtship behavior. *V1a1*^{F93Y} homozygote mutant males exhibited normal aggressive behavior (A) and courtship behavior (B). Mean ± SEM. Each n = 8 (A), n=11 (B), Mann-Whitney U-test.

	Mate-guarding (♂−♀−♂)		Aggression (♂ → ♂)	Courtship (♂ → ♀)
	Elicitation	Dominance		
vt $^{M1R/M1R}$	○	low	normal	low
V1a1$^{F93Y/F93Y}$	○	normal	normal	normal
V1a2$^{+/N68I}$	○	-	low	normal
V1a2$^{N68I/N68I}$	×	low	low	low

Table 1 Summary of mutant phenotypes.

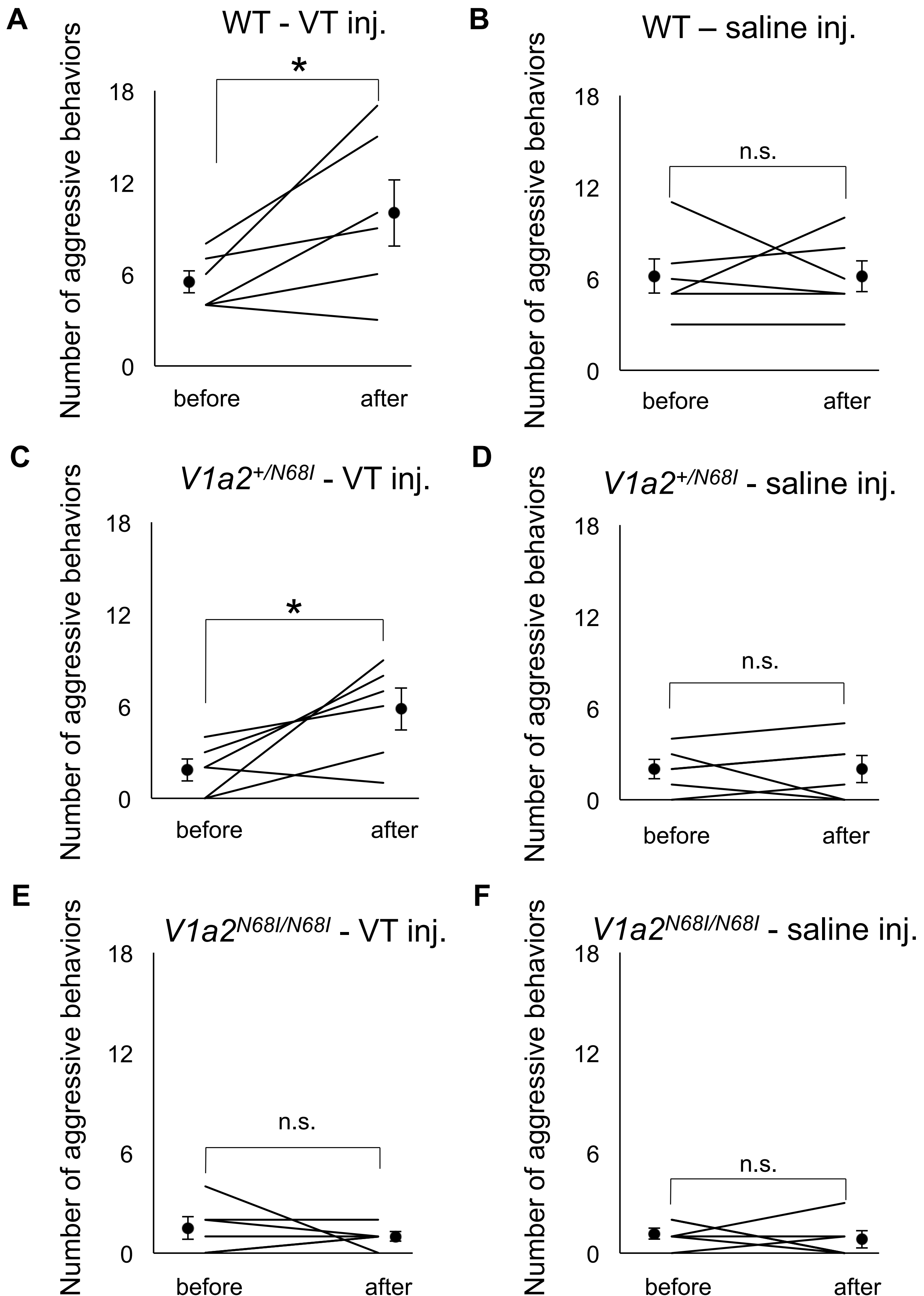
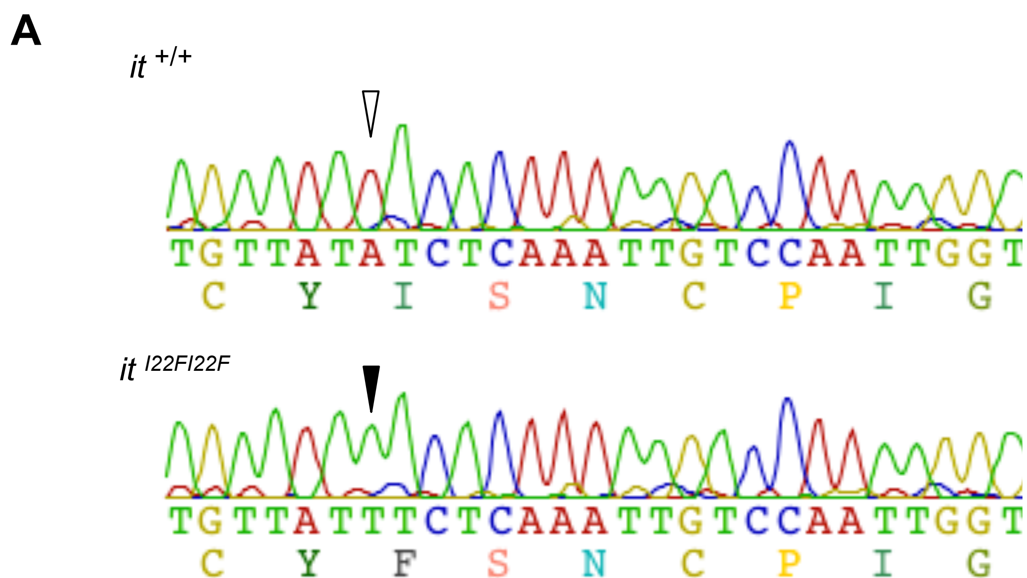


Figure 25 Effect of VT administration on aggressive behavior. VT or saline was injected to the one of three fish intraperitoneally and the number of aggressive behaviors of focal male toward other males before and after injection was counted. (A, C, E) Injection of VT increased aggression of wild-type males and *V1a2*$^{+/N68I}$ males (A, C). Injection of VT did not increase aggression of *V1a2*$^{N68I/N68I}$ males (E). Injection of saline did not alter aggression of the wild-type males (B), *V1a2*$^{+/N68I}$ males (D), and *V1a2*$^{N68I/N68I}$ males (F). Mean ± SEM. Each n = 6, Wilcoxon signed-rank test: *P<0.05 VS before.



B

species	gene	Amino acid sequence								
mouse	*oxytocin*	C	Y	I	Q	N	C	P	L	G
chicken	*mesotocin*	C	Y	I	Q	N	C	P	I	G
fugu	*isotocin*	C	Y	I	S	N	C	P	I	G
medaka	*isotocin*	C	Y	I	S	N	C	P	I	G
medaka	*it*^{I22F}	C	Y	F	S	N	C	P	I	G

Figure 26 Screening for *it* mutants by TILLING methods. (A) A local sequence dataset comparing the wild-type *it* (*it*$^{+/+}$) and it^{I22F} homozygotes (*it*$^{I22F/I22F}$) demonstrating the *it* A66T mutation in it^{I22F} mutants (black arrowhead). (B) The primary structures of *it* homologs in vertebrates. All peptide is composed of 9 amino acids. Isoleucine 20, which is identical among *it* homologs, was changed to phenylalanine in *it*^{I22F} mutant allele (red letters).

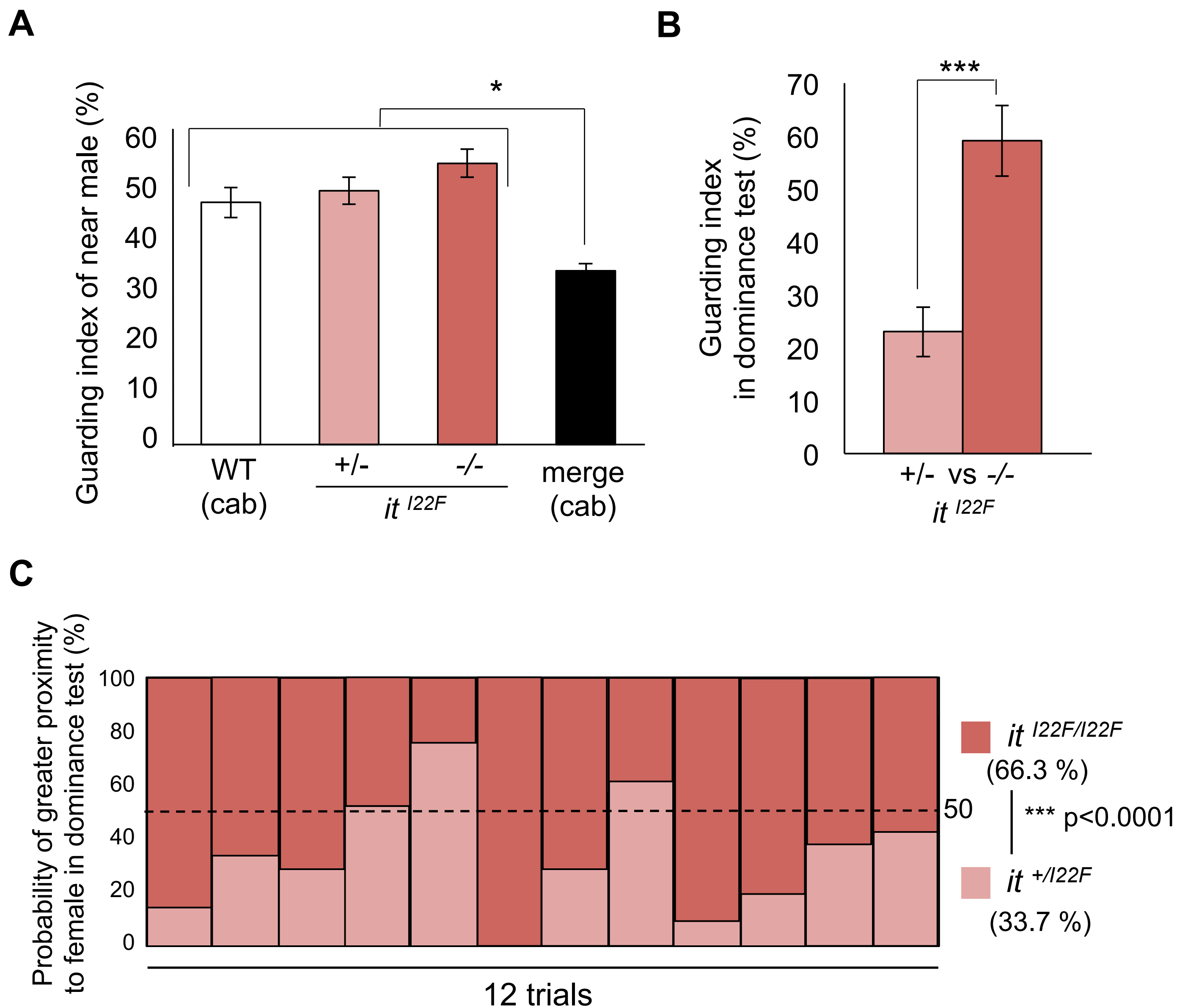


Figure 27 Mate-guarding behavior of *it* mutant males. (A) *it* ($it^{I22F/I22F}$) mutants exhibited mate-guarding in the guarding test. Mean ± SEM. Each n = 12, Dunnett's test : *P<0.05. (B) *it* homozygote mutants ($it^{I22F/I22F}$) tended to be dominant males in the dominance test. Mean ± SEM. Each n = 12, Student's t-test: ***P<0.001. (C) Dominant males in the mate-guarding behavior maintained closer proximity to the female than subordinate males. I judged which male was closer to the female in a total 256 images (21 images x 12 trials) in the dominance test. Here I calculated the probabilities of being closer to the female between *it* heterozygote and homozygote mutants based on the 256 images. I then detected a significant bias between these two probabilities using the chi-square test.

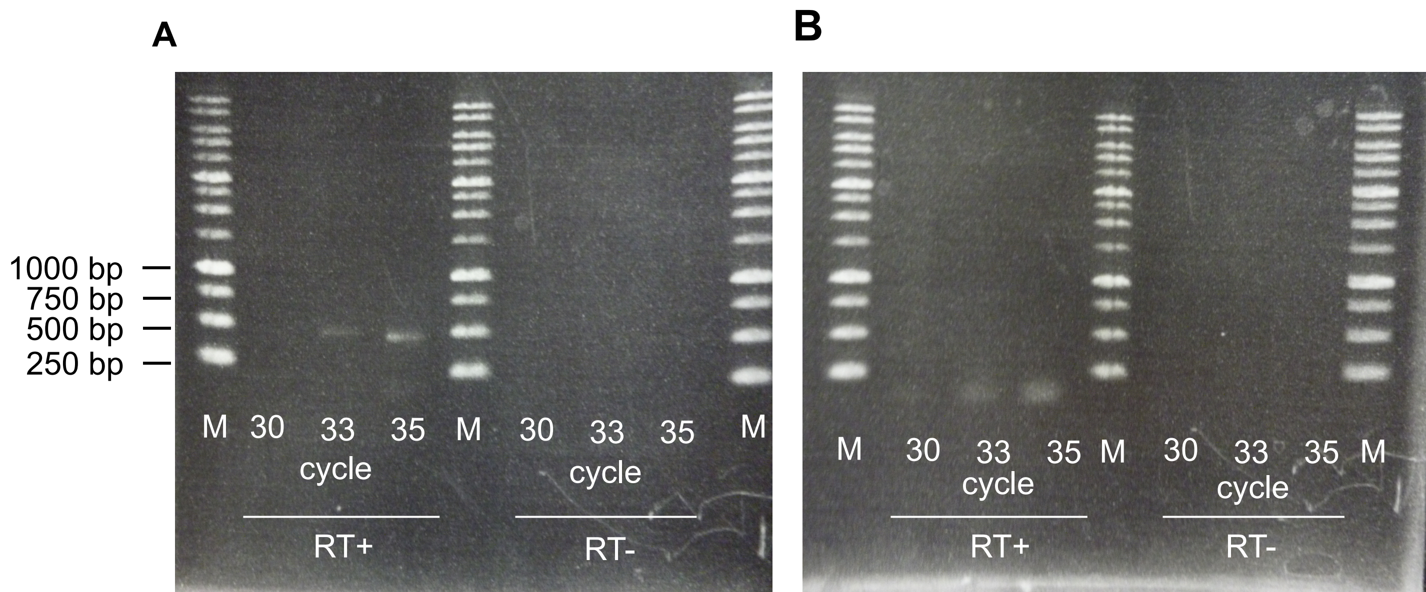
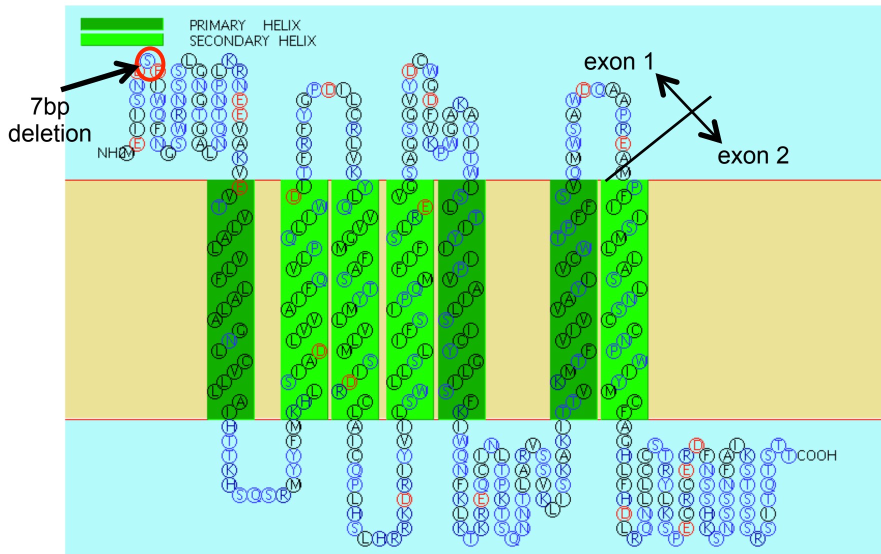
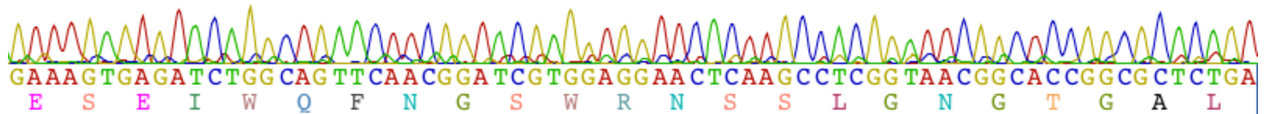


Figure 28 RT-PCR analysis of two genes for IT receptors in the medaka brain. (A) ITR1 mRNA was detected in the medaka brain. The estimated product size by PCR amplification was 450bp. (B) ITR2 mRNA wasn't detected in the medaka brain. The estimated product size by PCR amplification was 761bp.

A



ITR1$^{+/+}$



ITR1 KO (7bp deletion)

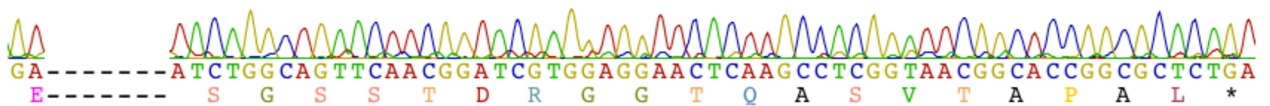


Figure 29 Generation of *ITR1* mutant alleles by TALEN. (A) A local sequence dataset comparing the wild-type *ITR1* (*ITR1*$^{+/+}$) and *ITR1* knockout (KO) homozygotes demonstrating that a 7-bp deletion generated a nonsense mutation (G27X) in *ITR1* KO mutants. *ITR1* gene consists of two exons. The deletion was located in the first exon and the mutated transcripts encode C-terminal deleted proteins lacked six of the seven transmembrane domains encoded by the first exon.

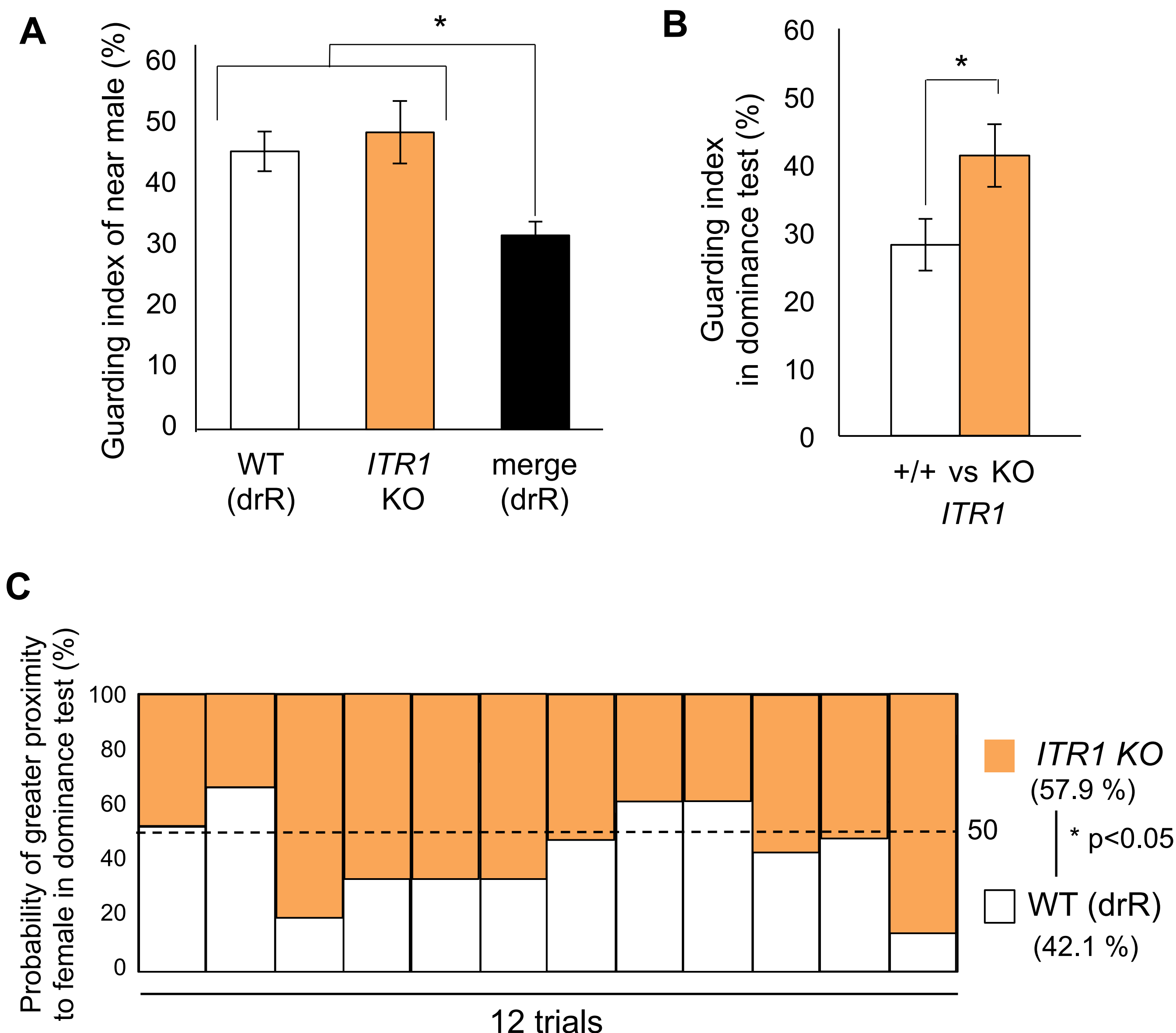


Figure 30 Mate-guarding behavior of *ITR1* mutant males. (A) *ITR1* KO mutants exhibited mate-guarding in the guarding test. Mean ± SEM. Each n = 12, Dunnett's test : **P*<0.05. (B) *ITR1* KO mutants tended to be dominant males in the dominance test. Mean ± SEM. Each n = 12, Student's t-test: ****P*<0.001. (C) Dominant males in the mate-guarding behavior maintained closer proximity to the female than subordinate males. I judged which male was closer to the female in a total 256 images (21 images x 12 trials) in the dominance test. Here I calculated the probabilities of being closer to the female between *ITR1* KO mutants and wild-type based on the 256 images. I then detected a significant bias between these two probabilities using the chi-square test.

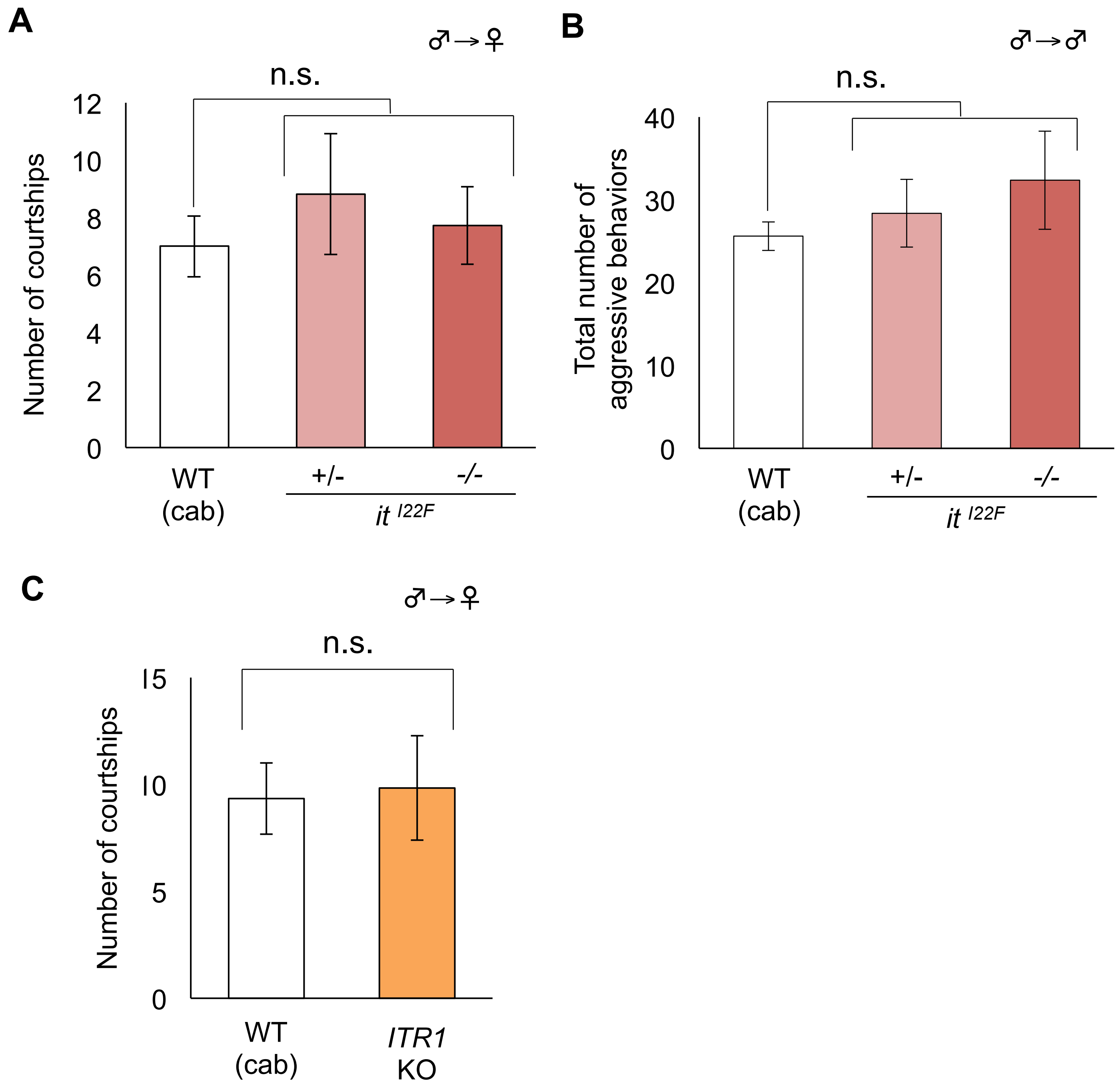


Figure 31 Effect of IT-related genes mutations on courtship behavior (intersexual interaction) and aggressive behavior (intrasexual interaction). (A) *it* mutant males exhibited courtship behaviors normally. Mean ± SEM. Each n = 11, Dunnett's test. (B) *it* mutant males exhibited normal aggression. Mean ± SEM. Each n = 8, Dunnett's test. (C)) *ITR1* mutant males normally exhibited courtship behaviors. Mean ± SEM. Each n = 11, Dunnett's test.

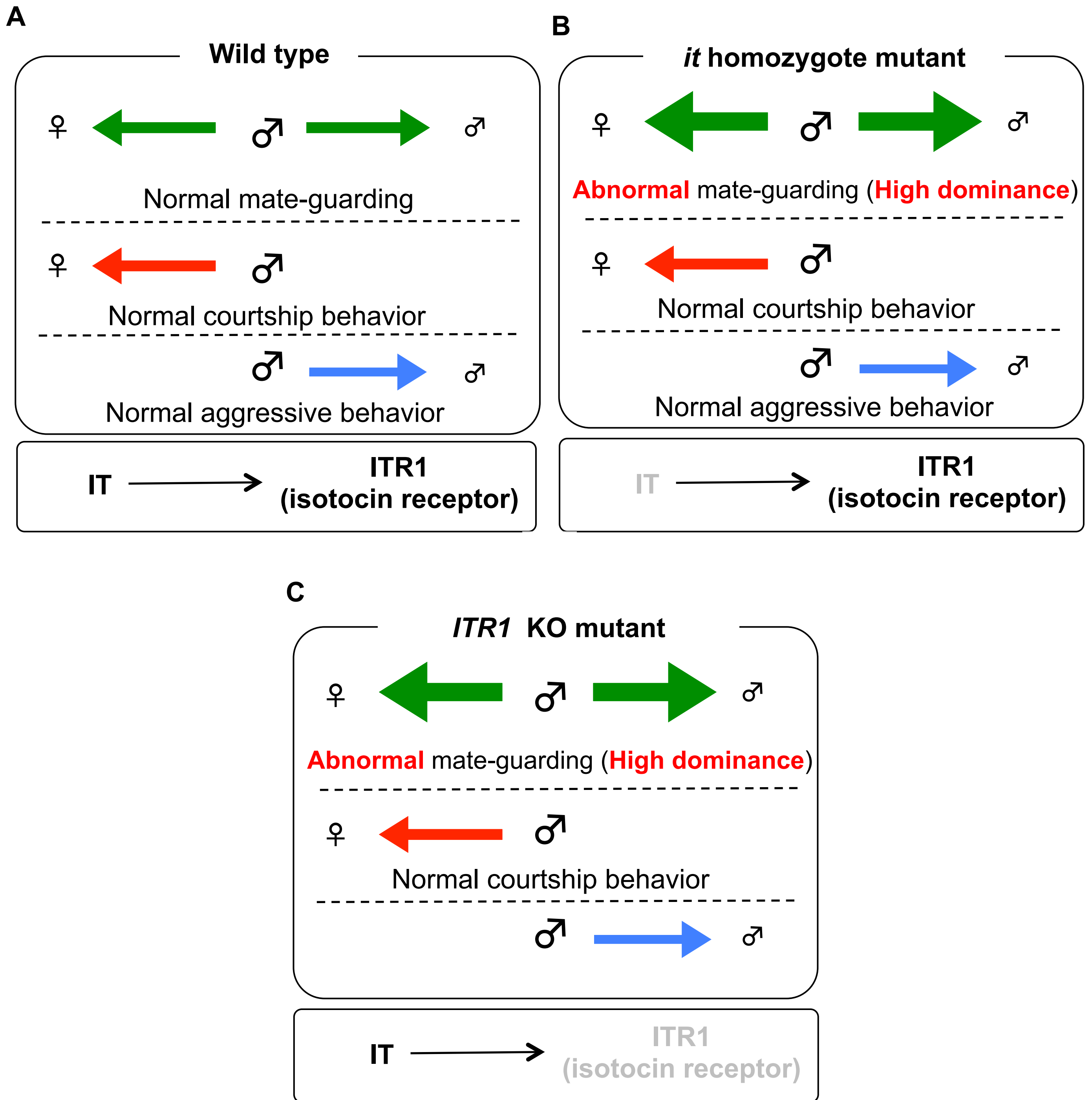


Figure 32 Summary of behavioral phenotype and related IT system. (A) In addition to the sexual motivation toward the opposite sex (red arrow) and competitive motivation toward the same sex (blue arrow), social motivation enhanced only in the triadic relationship (green arrow) regulate mate-guarding. IT system might suppress this third motivation. (B) The homozygote *it* mutant males normally have sexual motivation and competitive motivation. High dominance in mate-guarding was caused by low suppression of the social motivation enhanced only in the triad (bold green arrow). (C) The *ITR1* KO mutant males normally have sexual motivation. Based on the behavioral result of homozygote *it* mutant males, high dominance in mate-guarding might be caused by low suppression of the social motivation enhanced only in the triad (bold green arrow).

	Mate-guarding (♂−♀−♂)		Aggression (♂ → ♂)	Courtship (♂ → ♀)
	Elicitation	Dominance		
vt $^{M1R/M1R}$	○	low	normal	low
V1a1$^{F93Y/F93Y}$	○	normal	normal	normal
V1a2$^{+/N68I}$	○	-	low	normal
V1a2$^{N68I/N68I}$	×	low	low	low
it $^{I22F/I22F}$	○	high	normal	normal
ITR1 KO	○	high	-	normal

Table 2 Summary of mutant phenotypes (Chapter 2 & 3).

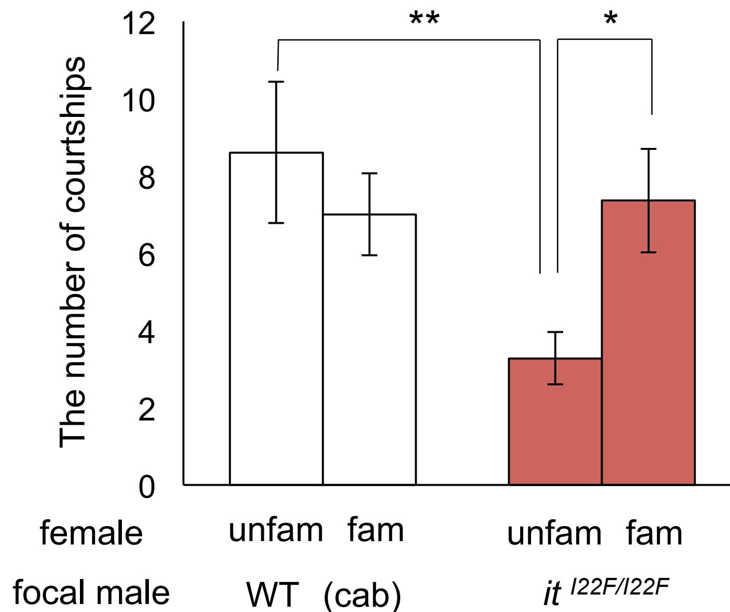


Figure 33 Courtship behavior toward socially unfamiliar or familiar females. WT(cab) males exhibited courtship behavior toward unfamiliar females (*it* $^{I22F/I22F}$ females) to the same extent as toward familiar females (WT females). In contrast, *it* (*it* $^{I22F/I22F}$) mutant males exhibited courtship behavior toward unfamiliar females (WT females) less frequently than toward familiar females (*it* $^{I22F/I22F}$ females). "unfam"…unfamiliar female. "fam"…familiar female. Mean ± SEM. Each n = 11, Tukey-Kramer's test: *P<0.05, **P<0.01

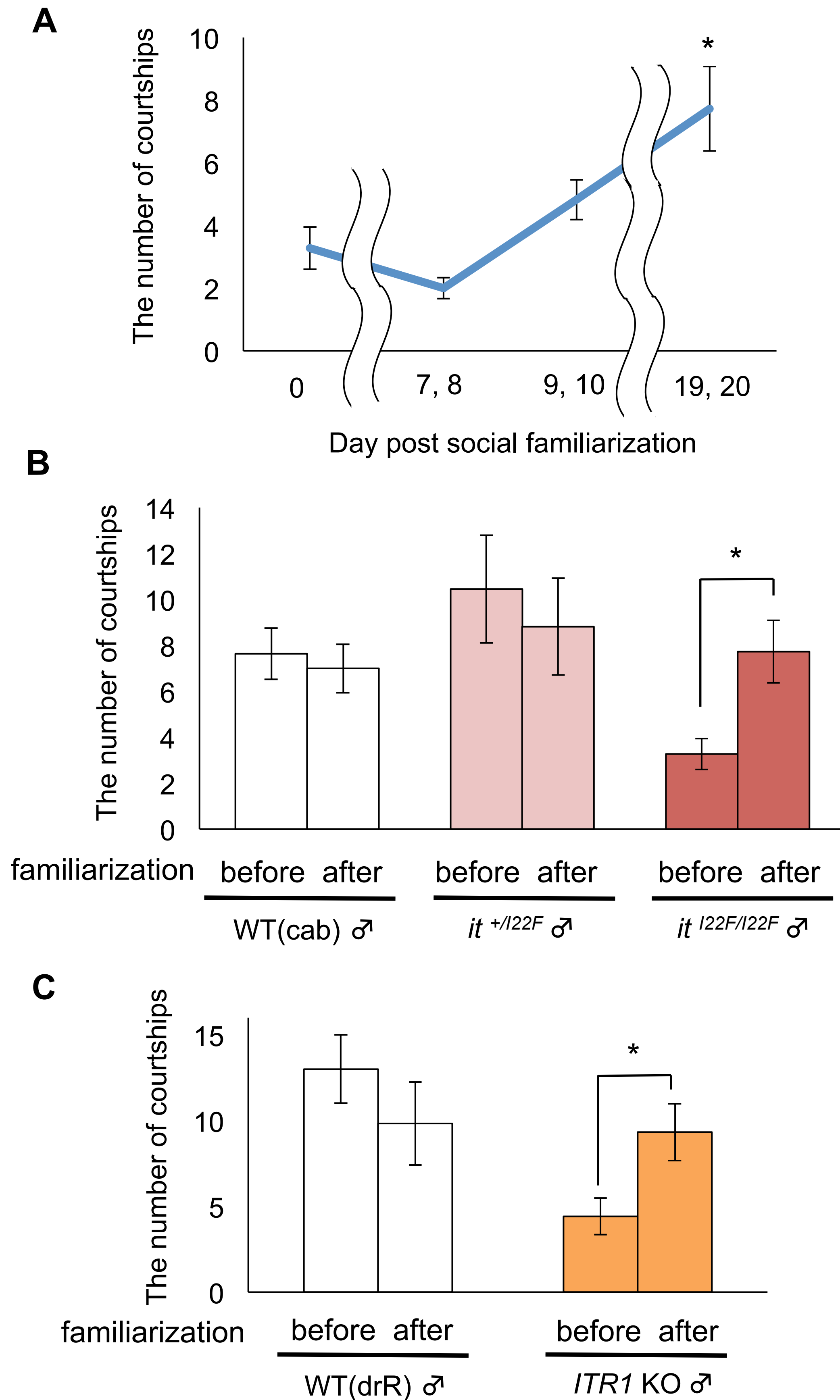
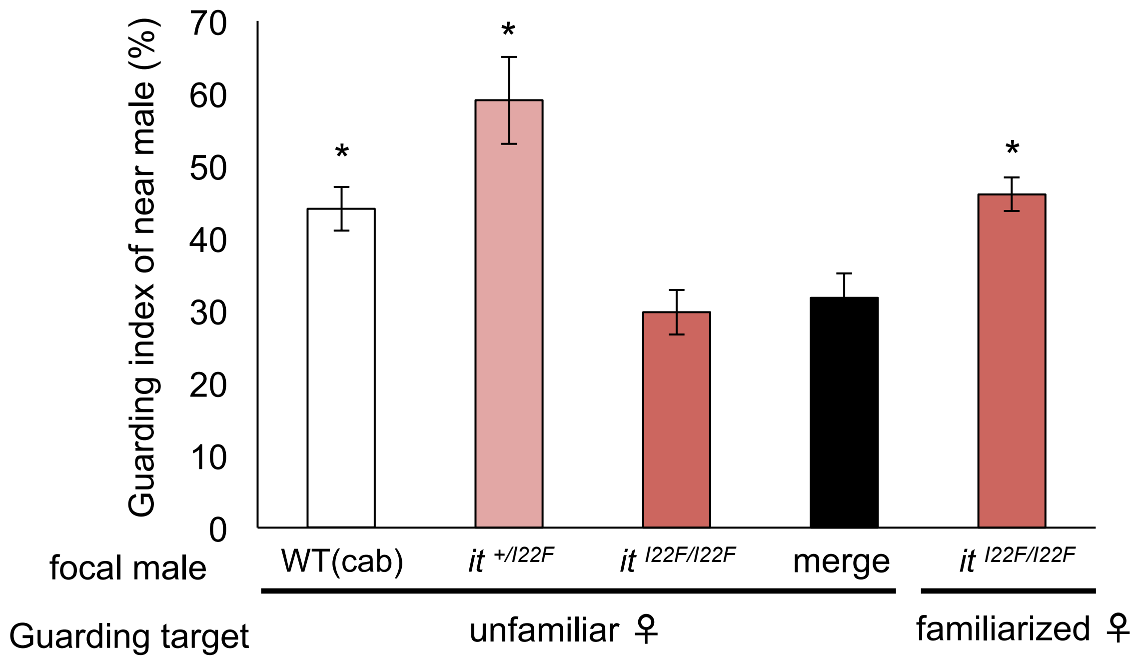


Figure 34 Effect of social familiarization on the courtship behavior. (A) The number of courtship behaviors of *it* homozygote mutant males toward unfamiliar WT females was significantly increased by 19-20 days social familiarization. Mean ± SEM. Each n = 11, Dunnett's test: *P<0.05 VS "0". (B) 19-20 days social familiarization did not affect the sexual motivation of WT and *it* heterozygote mutant males. Mean ± SEM. Each n = 11, Student's t-test: *P<0.05. (C) The low sexual motivation of *ITR1* KO mutant males toward unfamiliar WT females was recovered by 19-20 days social familiarization. Mean ± SEM. Each n = 11, Student's t-test : *P<0.05.



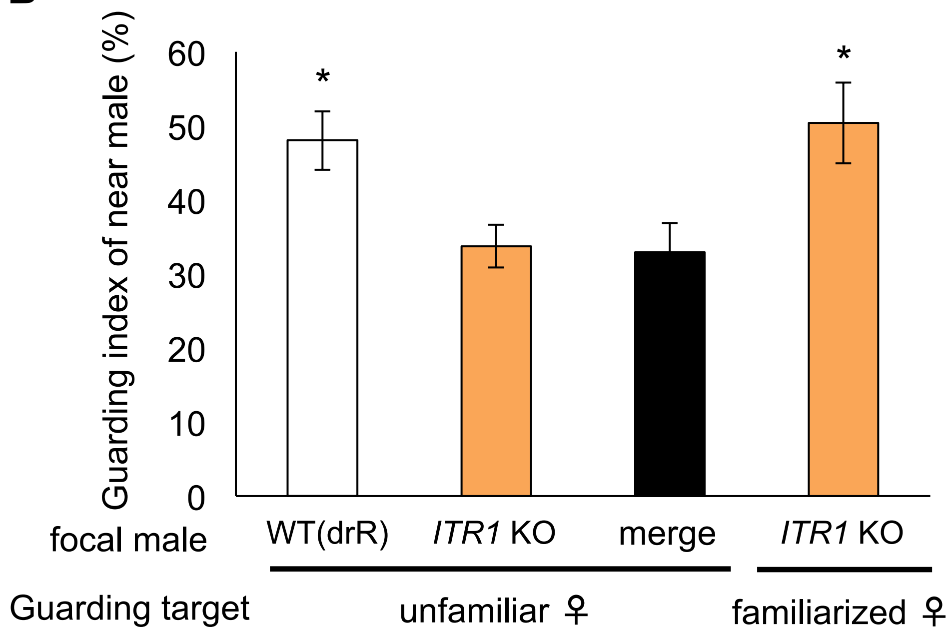


Figure 35 Mate-guarding behavior of *it* and *ITR1* mutant males toward socially unfamiliar and familiarized females. (A) WT males exhibited mate-guarding even toward unfamiliar females. In contrast, *it* homozygote mutant males exhibited mate-guarding only toward familiarized females, and not toward unfamiliar females. Mean ± SEM. Each n = 12, 10, 12, 13, 12, respectively. Dunnett's test: $*P<0.05$ VS "merge" (B) *ITR1* mutant males exhibit mate-guarding only toward familiarized females, and not toward unfamiliar females. Mean ± SEM. Each n = 12, Dunnett's test: $*P<0.05$ VS "merge"

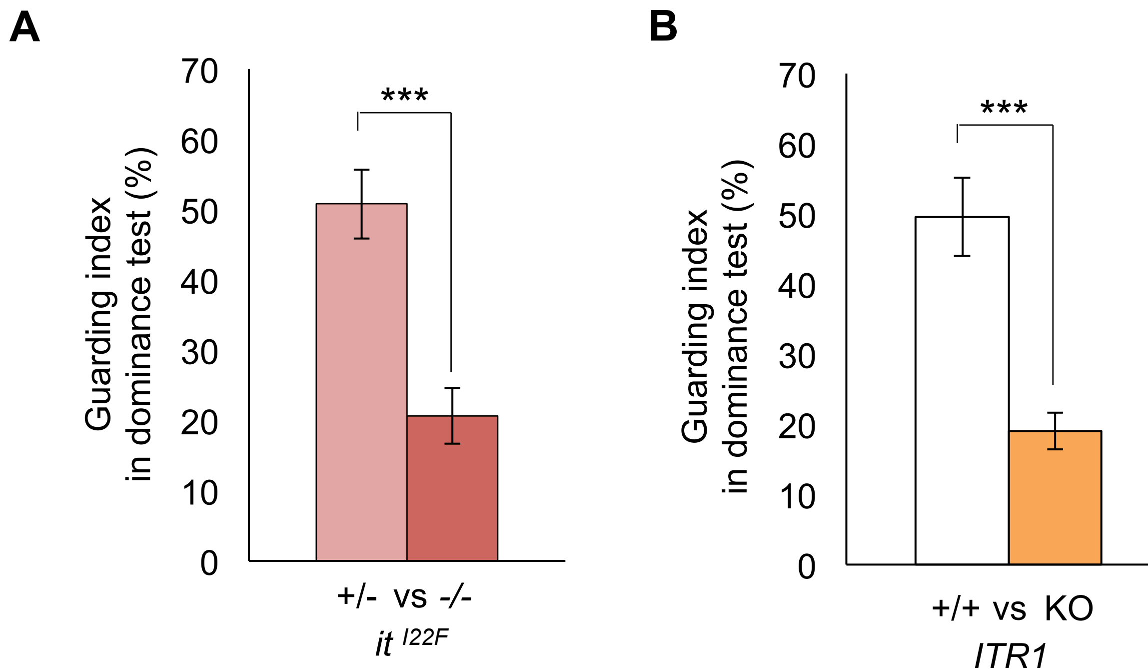
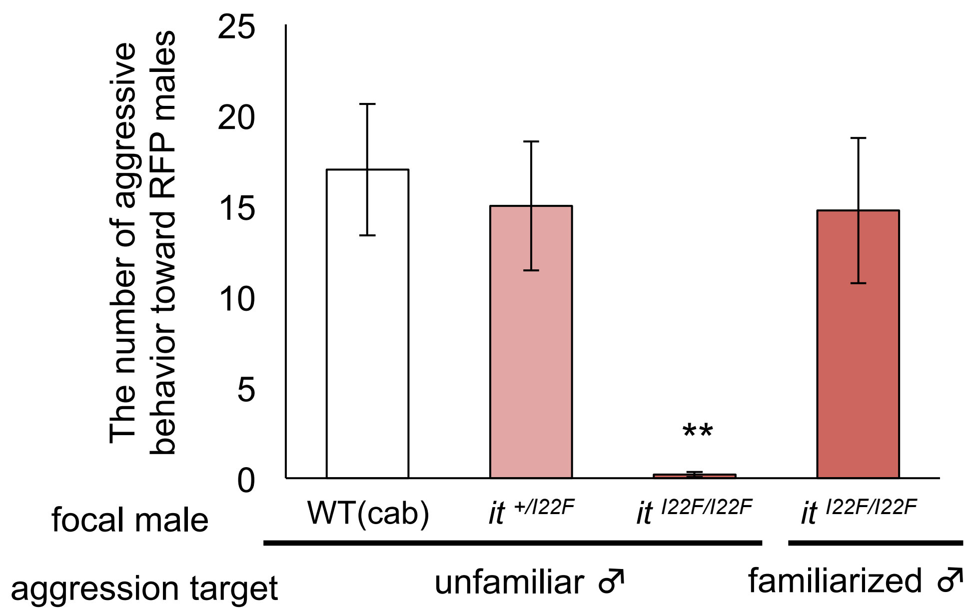


Figure 36 Dominance test using socially unfamiliar females. (A) *it* homozygote mutant males (*it* $^{I22F/I22F}$) tended to be subordinate in the dominance test using socially unfamiliar females. Mean ± SEM. Each n = 12, Student's t-test: ****P*<0.001. (B) *ITR1* KO mutant males tended to be subordinate in the dominance test using socially unfamiliar females. Mean ± SEM. Each n = 12, Student's t-test: ****P*<0.001.



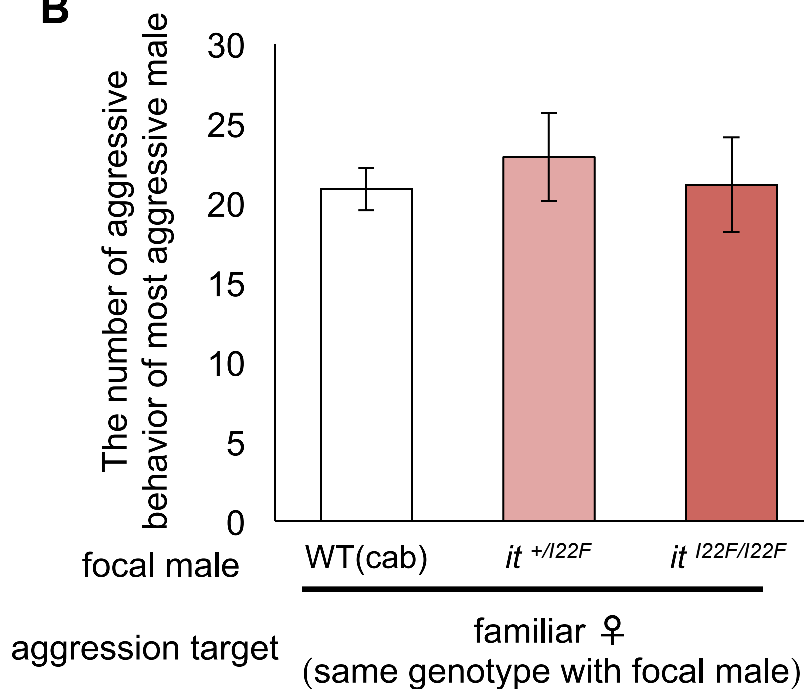


Figure 37 Aggressive behavior of *it* and *ITR1* mutant males toward socially unfamiliar, familiarized, and familiar males. (A) WT males exhibited aggressive behavior even toward unfamiliar RFP males. In contrast, *it* homozygote mutant males exhibited aggressive behavior only toward familiar males, and not toward unfamiliar males. Mean ± SEM. Each n = 10, 10, 10, 8, respectively. Dunnett's test: $^{**}P<0.01$ VS "WT" (B) *it* homozygote mutant males exhibited aggressive behaviors toward familiar males with the same genotype as the focal males, to the same extent as WT and *it* heterozygote mutant males. Mean ± SEM. Each n = 8, Dunnett's test.

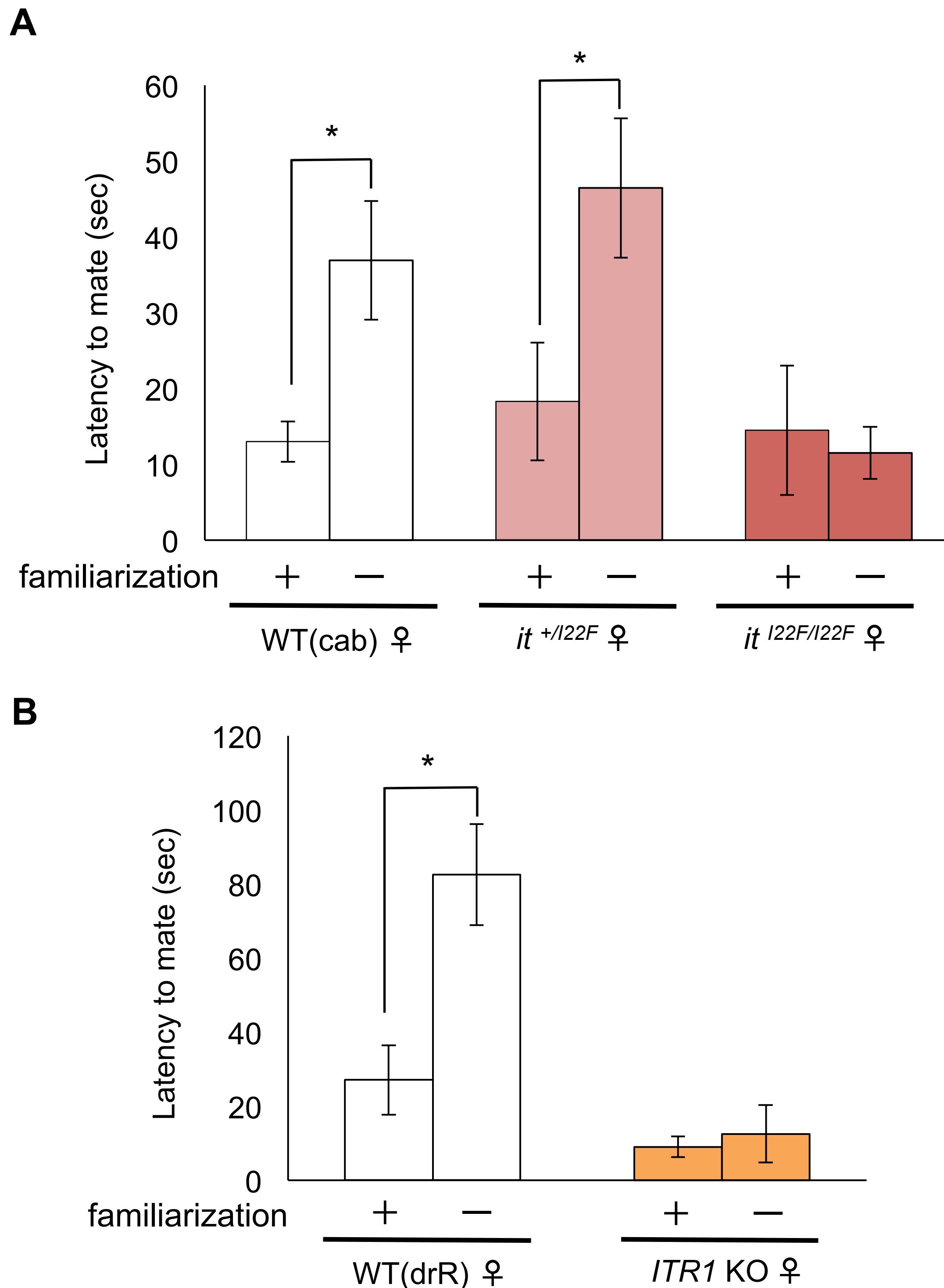


Figure 38 Female mate preference of *it* and *ITR1* mutant males. (A) In *it* homozygote mutant females, the latency to mate with unfamiliar males was as short as that with visually familiarized males. Mean ± SEM. Each n = 10, Student's t-test: *P<0.05. (B) In *ITR1* KO mutant females, the latency to mate with unfamiliar males was as short as that with visually familiarized males. Mean ± SEM. Each n = 10, Student's t-test: *P<0.05.

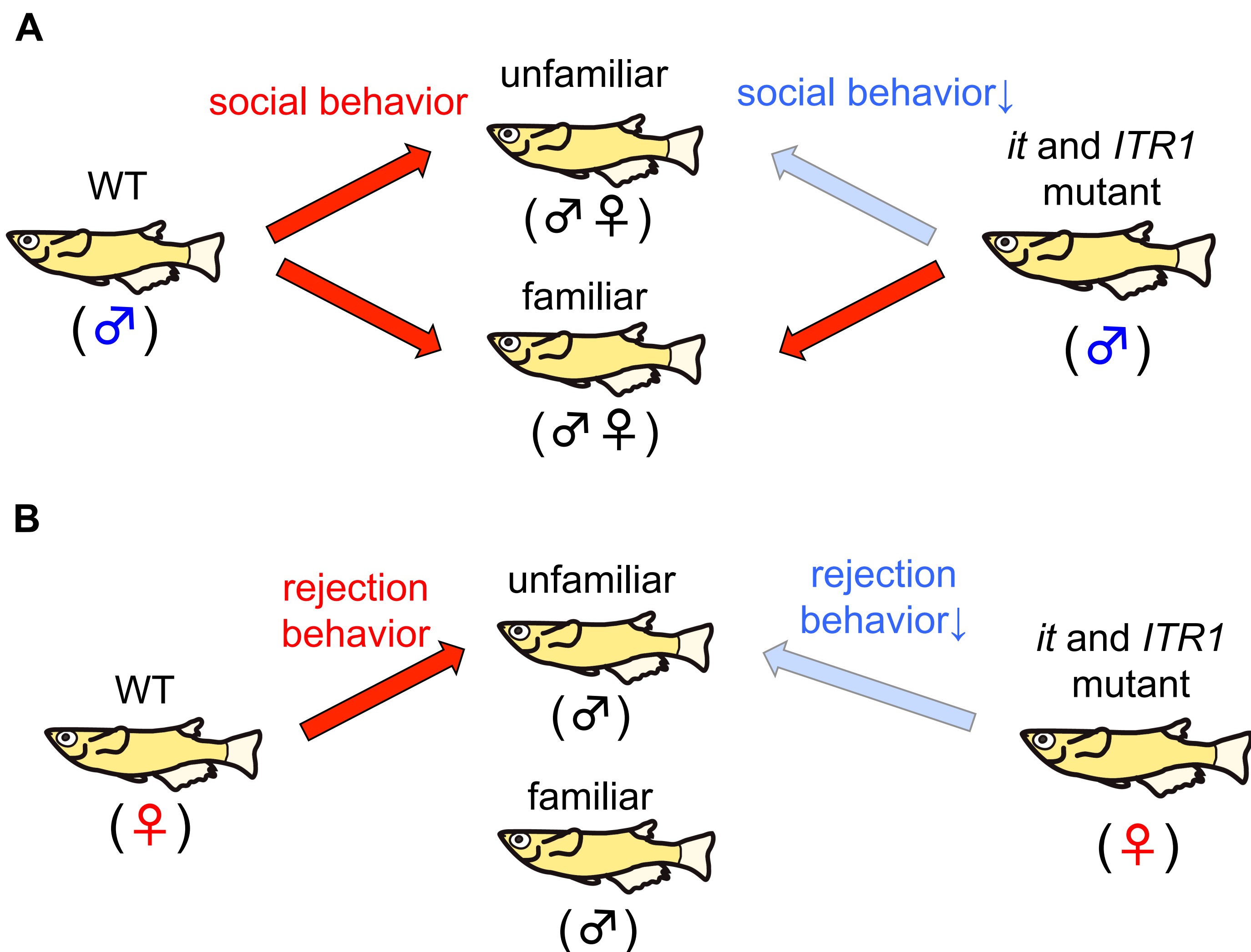


Figure 39 Summary of behavioral phenotypes based on social familiarity in *it* and *ITR1* mutant males. (A) WT males exhibited social behaviors (courtship, mate-guarding and aggressive behavior) irrespective of social familiarization. In contrast, *it* and *ITR1* mutant males hardly exhibited social behaviors toward socially unfamiliar fish, while they exhibited these behaviors toward socially familiar fish. (B) WT females rejected the courtship behavior of socially unfamiliar males. In contrast, *it* and *ITR1* mutant females hardly exhibited such rejection behavior and quickly mated with unfamiliar males.

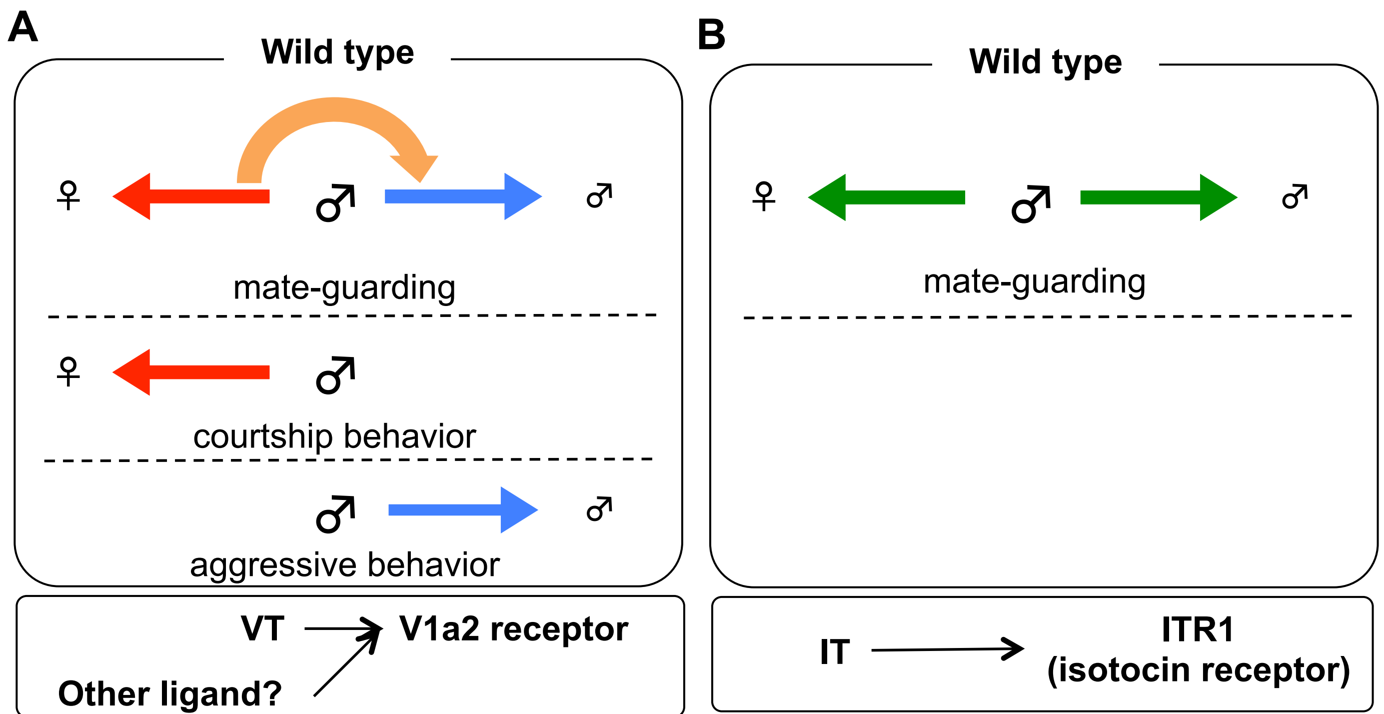


Figure 40 Independent regulation model of mate-guarding via the VT system and IT system. (A) The VT system regulates male social behaviors (mate-guarding, courtship, and aggressive behaviors) and the presence of a potential mating partner may induce competitive motivation toward rival males via the VT system, leading to male-male competition (orange arrow). (B) The IT system is required for normal mate-guarding, but not for dyadic behaviors (courtship and aggressive behaviors). The IT system may suppress social motivation enhanced only in the triadic relationship (green arrow), which regulates mate-guarding. The regulation pathways of mate-guarding by the VT and IT systems may be independent.

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