A behavioral study on the effect of mating experiences with the same partner on mating activities of males and females in medaka fish

博士論文

A behavioral study on the effect of mating experiences with the same partner on mating activities of males and females in medaka fish

(メダカにおける同一パートナーとの性行動の経験が雌雄の性行動の活性に与える行動学的研究)

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東北大学大学院生命科学研究科

脳生命統御科学専攻

大門　将寛

Content

Abbreviations

BNST:	Bed Nucleus of the Stria Terminalis
cDNA:	complementary DNA
DEG:	Differentially Expressed Genes
DIO2:	Iodothyronine Deiodinase 2
EC:	Embryonic Carcinoma
FDR:	False Discovery Rate
GLMM:	Generalized Linear Mixed Model
LC-MS/MS:	Liquid Chromatography – Mass Spectrometry
mPOA:	Medial Preoptic Area
NPP:	Nucleus Praeopticus Periventricularis
RNA-seq:	RNA sequencing
T3:	Triiodothyronine
T4:	Tetraiodothyronine or Thyroxine
VGF:	VGF nerve growth factor inducible
Vp:	Supracommissural and Nuclei of the Ventral Telencephalic Area
Vs:	Posterior Nuclei of the Ventral Telencephalic Area

Abstract

The roles of mating and social experience in shaping male mating behaviors have been compared across species since the 1950's. Many similarities and differences exist among species in the effects of mating experiences on the motivational and performance aspects of mating behaviors. For example, in fruit flies (*Drosophila melanogaster*), the first mating experience in males shortens the latency to the first courtship with a female. In rodents such as mice (*Mus musculus*) and rats (*Rattus norvegicus*), the first male mating experience decreases the latency to the first intromission. In fish species such as the blue gouramis (*Trichogaster trichopterus*), similar effects of repeated mating experiences are also observed. In addition, mating experience activates mate preference in mosquitofish (*Gambusia holbrooki)*; Vega-Trejo et al. quantified mate preference in mosquitofish, and demonstrated that the amount of time spent with a novel female was significantly increased by the mating experience, but not by visual and olfactory familiarization. These studies indicated that the male mating experience could facilitate male mating activities and influence male mate preference.

In rodents, the behavioral change triggered by the first mating experience is associated with changes in the neural circuits and substrates in the brain. The number of neurons in the olfactory bulbs and the density of mushroom spines in the medial preoptic area (mPOA) are increased by the first mating experience in mice. No studies to date have revealed the neural/molecular mechanisms underlying behavioral changes dependent on the mating experience in fish species. To address this question, I used medaka fish (*Oryzias latipes*) in the present study. There are many advantages to using medaka fish for studying mating behavior. First, medaka mating behavior

comprises several steps (approach, courtship display, wrapping, and spawning), which allows for the quantification of male mating activity under laboratory conditions. Second, as the female reproductive cycle is 24 h, and therefore the same female ready to spawn can be used for mating tests every morning. Third, medaka is a model animal for molecular genetics, and state-of-the-art molecular genetic techniques are available.

In chapter 1, to investigate how the mating experience of naïve males could influence mating behavior in medaka fish, I compared mating behaviors between naïve and experienced males. To prepare naïve males, I separated juvenile males into groups and bred them without any females until performing the mating tests. I also prepared sexually mature females for the mating test. I performed mating tests in fixed dyads in 7 continuous mating tests, revealing that the latency to mate significantly decreased after the mating experience and biased the time-dependent change of the latency to mate only in naïve males and not in experienced males. Next, I compared the latency to the first courtship display between naïve and experienced males, revealing that the latency to the first courtship display significantly decreased after the first mating experience in naïve males, suggesting male mating activity increased after the mating experiences. Furthermore, I examined whether the mating experience with the naïve males altered female mating behavior. I compared the latency to mate after the first courtship display. This index negatively correlated with the degree of female receptivity, because females with high receptiveness tend to accept males immediately after the first courtship display. The latency was significantly decreased in naïve males, suggesting that the female mating experience with the naïve males enhanced female receptivity. In short, I revealed the male first mating experience altered both male and female mating behaviors. Finally, to examine whether this effect was specific to fixed dyads, I performed another mating tests in swapped dyads in naïve males. The latency to mate and to the first courtship

display were significantly decreased only in the fixed group, showing that the naïve males recognized the first mating partner, and thus the latency to mate was decreased according to the mating experience.

In chapter 2, to evaluate the effect of the mating experience in naïve males on the brain gene expression patterns, I compared gene expression profiles of whole brains (including the pituitary) between the naïve and post-naïve male medaka that had 2 mating experiences. As results, interestingly, I found that 3 genes (*tshba*, *dio2*, *klf9*) of the top 10 DEGs (differentially expressed genes) were associated with functional expression of the thyroid hormone system. Therefore, I conducted measurements of the thyroid hormone in naïve and post-naïve with two mating experiences male brains using the LC-MS/MS technique. The average amount of the activated thyroid hormone T3 was increased 1.17 times in post-naïve samples compared with naïve samples, however there was no significant difference. Therefore further study is needed with multiple time points to reveal the dynamics of the thyroid hormone in naïve male brain.

In this work, I revealed that the mating experience can alter the male mating activity to the familiarized females in naïve medaka males. Interestingly, the pattern of behavioral alterations in medaka fish was quite different from those of other species such as fruit flies, mice, and rats, in which the mating experience facilitates male mating activity to any females but does not influence mating activity toward familiar females. What was the adaptive significance of the enhanced mating activity in naïve males toward familiar females? One possibility is that under an environmental condition where males have few chances to encounter females, males might enhance mating/courtship activity toward females that he had mated once, which could activate female mate preference of the target female by familiarization. In addition, I also revealed mating experiences up-regulated 3 genes that are required for a functional cascade of the thyroid hormone

system in the naïve male brain. This is the first finding to imply the activation of the thyroid hormone system depending on the male mating experiences in the naïve males. Thyroid hormone contributes to the dendritic spine maturation in mice brain via transcription factor *kllf9* in mice. Therefore, the mating experience in naïve males may change the neuronal morphology according to thyroid hormone levels in male medaka.

General introduction

The roles of mating and social experience in shaping male mating behaviors have been compared across species since the 1950's [1]. Many similarities and differences exist among species in the effects of mating experiences on the motivational and performance aspects of mating behaviors [2–5]. For example, in fruit flies (*Drosophila melanogaster*), the first mating experience in males shortens the latency to the first courtship with a female [2]. Furthermore, in a competitive mating situation between 1 female, a naïve male, and an experienced male, the experienced males more frequently exhibit abdominal bends (attempted copulation) than naïve males. In rodents such as mice (*Mus musculus*) [3,4] and rats (*Rattus norvegicus*) [5], the first male mating experience decreases the latency to the first intromission, i.e., mounting behavior with penis insertion. In fish species such as the blue gouramis (*Trichogaster trichopterus*), similar effects of repeated mating experiences are also observed [6]. In birds such as Japanese quail (*Coturnix japonica*) [7] and ring doves (*Streptopelia risorii*) [8], naïve males usually approach the females, but some of them fail to copulate on their first encounter. By their second encounter, however, most males successfully complete copulation behaviors in a species-specific manner [7,8]. In addition, mating experience activates mate preference in mosquitofish (*Gambusia holbrooki)*; Vega-Trejo et al. quantified mate preference in mosquitofish using a 3-chamber test, and demonstrated that the amount of time spent with a novel female was significantly increased by the mating experience, but not by visual and olfactory familiarization [9].

In rodents, the behavioral change triggered by the first mating experience is associated with changes in the neural circuits and substrates in the brain. The number of neurons in the olfactory

bulbs and the density of mushroom spines in the medial preoptic area (mPOA) are increased by the first mating experience in mice [4,10]. In contrast, the mushroom spine density in the mPOA decreases and the expression of *vgf,* which encodes the neuropeptide precursor VGF in the mPOA, affects behavior (shortening mating latency) following mating and ejaculation experience in male rats [5,11]. A recent study revealed that the expression of gastrin-releasing peptide and oxytocin receptors is increased in the spinal ejaculation generator in the lumbosacral cord after the first mating experience with ejaculation in male rats [12]. Furthermore, the first mating experience also decreases neuronal activity in the center part of the mPOA, suggesting that the first mating experience reconstructs the neural network associated with male mating behavior [13]. No studies to date have revealed the neural/molecular mechanisms underlying behavioral changes dependent on the mating experience in fish species.

In the present thesis, I showed that repeated mating experiences shortened the latency to mate as well as that to the first courtship only in naïve males not in experienced males in medaka (Chapter 1). Furthermore, I revealed this behavioral change occurred when naïve males mated repetitively with the same females, suggesting naïve males could have ability to recognize specific females. In addition, female sexual receptivity increased when females mated with specific naïve males. Next, to identify genes whose expression is regulated by the mating experience in the naïve male brain, I compared gene expression profiles before/after mating experience in naive males using next generation sequencer technique (Chapter 2). I found that 3 genes which are related to the functional expression of the thyroid hormone system were up-regulated after the mating experience in naïve male medaka. To my knowledge, this is the first finding to clarify the thyroid hormone system might be activated by the mating experience in the naïve male.

Chapter1

Behavioral experiments to reveal the effect of mating experiences in naïve male medaka

Introduction

In the general introduction, I mentioned that no studies have revealed the brain molecular mechanism underlying the alteration of male mating behaviors according to the mating experience of naïve males in fish species. To approach this issue, I decided to establish the behavioral assay to measure the effect of the mating experience on male mating activity using model animals in the field of molecular genetics. Therefore, I used medaka fish (*Oryzias latipes*) in the present study. There are many advantages to using medaka fish for studying mating behavior. First, medaka mating behavior comprises several steps (approach, courtship display, wrapping, wrapping rejection and spawning). In addition, a mutant strain, whose body color is different between sexes, is available for mating tests, which allows for quantification of sex-specific behaviors under laboratory conditions easily [14]. Second, as the female reproductive cycle is 24 h, the same female ready to spawn can be used for mating tests every morning [15]. Therefore, I thought that medaka is one of the most suitable animals to validate the male mating plastically change.

In this chapter, I established the behavioral system to compare the male mating activity between naïve and sexually-experienced males in medaka. First, I examined how the mating experiences could change mating activities using naïve and experienced males. Second, I validated whether the mating experiences altered female mating receptivity, which is negatively correlated with the latency to mate after the first male courtship. Third, I tested whether the familiarization with the same females induced the behavioral change or not.

Materials & Methods

Ethics statement

All the methods in this study were carried out in accordance with relevant guidelines and regulations. The work in this paper was conducted using protocols specifically approved by the Animal Care and Use Committee of Okayama University (permit number: OKU-2015467) and Tohoku University (permit number: 2022LsA-003). Surgical dissection of the brain to extract total RNA was performed under deep anesthesia using ice, and all efforts were made to minimize suffering following the NIH Guide for the Care and Use of Laboratory Animals Fish and breeding conditions. The study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines).

Animal maintenance

All fish (*Oryzias latipes*; d-rR strain) were bred in our laboratory. Fish larvae were fed Paramecium or small pellet foods (Hikari lab., Meito system, Japan or Medaka no Mai Next, Kyorin, Japan), juveniles were fed small pellet foods (Medaka no Mai Next), and adult medaka were fed pellet foods (TetraMin, Tetra, Germany or Otohime B2, Marubeni Nisshin Feed, Japan) a few times a day. Juvenile and adult fish were fed brine shrimp once a day. Medaka were maintained in groups in plastic aquariums (13 cm x 19 cm x 12 cm height) or polypropylene containers (48 cm x 36 cm x 20 cm height). The water temperature was maintained at 24–28˚C with white LED lights (Eco-slim, OHM ELECTRIC INC, Japan) for 14 h per day (08:00–22:00).

Animal preparation for mating tests

Adult male (>5 months of age) and female (>3 months of age) fish were used for this experiment. To prepare “naïve males”, I separated sexually immature males from females 1–2 months after hatching. I determined their sexes based on the body color difference[16] and fin shape. I used sexually matured females that had spawned fertilized eggs continuously for at least 3 days as sexually matured ready-to-spawn eggs. In the present study, I defined “naïve” males as sexually inexperienced and “experienced males” as adult males that had mated with females more than 7 times.

Mating test using fixed dyads

The mating test was performed as previously described[17]. To separate the male from the female, a plastic cup (CE-300, Kenis, Japan, 37 mm [radius] x 90 mm [height]) with white opaque paper was used from the night to the next morning (16:00–10:00). The day before the mating test, the opaque plastic cup with the naïve or experienced male was placed into a tank containing a female. The next morning, a male was released into the tank containing the female, which allowed them to begin their mating behavior (9:30–10:30). I recorded their mating behavior for 15 min using a Web camera (BSW200MKB, Buffalo, Japan). I repeated the mating tests for 7 days (times) using the same dyads. If a female did not spawn at least once over the 7 days, I excluded all data from the analysis. I manually measured the timing of the courtship display (male quick-circle dance), wrapping (crossing each body), wrapping rejection (wrapping with no spawning), and spawning by viewing the video, and calculated the latency to mate (period from releasing the male to the wrapping with spawning), the latency to the first courtship display, and the latency to mate after the first courtship. Transition probabilities (courtship from courtship [c -> c], wrapping from courtship [c -> w], courtship from wrapping rejection [wr -> c], and wrapping rejection from

wrapping [w -> wr]) were calculated by dividing the number of each behavioral transition by the total number of transitions. Experienced males who had mated with females more than 7 times were used for the same experiments as a control for 7 or 3 days (times). I also analyzed swimming distances of males and females during mating behavioral tests in day1 and day5. Firstly, I trimmed medaka mating videos (11 naïve males and 12 experienced males) from releasing males to wrapping with spawning and extracted picture frames per 5 sec using ffmpeg (version 4.2.2). Next, I measured centroid positions of males and females manually using ImageJ (version 2.3.0) and calculated the total transfer distances of males and females using R (version 4.0.5).

Mating test using swapped dyads

To determine whether the mating experience with the same partner was essential for changing the mating behavior, I performed the mating test using fixed and swapped dyads using naïve males continuously for 3 days (times). The procedure was the same as for the mating test described above.

Three chamber tests

I performed the three-chamber test according to the previous study (Yamashita et al., unpublished). Custom ordered transparent plastic aquarium (13.5 cm width x 19 cm x 12 cm height, water depth = 2 cm) was divided into 3 chambers using transparent acrylic plates (right and left compartment = 3.5 cm, center compartment = 6.5 cm). Subjected naïve male was transferred to the center compartment, and familiar naïve male that was maintained in the same group of subjected male and unfamiliar naïve male were to the left or right chamber respectively. I recorded this test using a Web camera (BSW500MBK, Buffalo, Japan) from the top side of the aquarium for 5 min after at least one minute for the habituation. I used UMAtracker[18] for the tracking. I calculated

individual distances between familiar and unfamiliar males ,and the mean individual distance of familiar males and unfamiliar ones were compared by paired T-test using default function of R.

Statistical analysis

Statistical analysis was run by R (version 4.0.5) with generalized linear mixed models (GLMMs) by the "glmer" function in the package lme4 (version 1.1-27) to reveal whether the number of matings (experience) affected medaka mating behavior. The gamma distribution (latency to mate, latency to the first courtship display, and latency to mate from the first courtship, swimming distance) and Poisson distribution (numbers of each event; courtship displays, wrappings, wrapping rejections, and transition probabilities of each behavioral transition; courtship from courtship [c -> c], wrapping from courtship [c -> w], courtship from wrapping rejection [wr -> c], and wrapping rejection from wrapping [w -> wr]) with a log link function were used for each statistical analysis. Transition probabilities were analyzed using the offset function in lme4 (eg., model = event numbers ~ mating times + type (naïve or experienced) + type : mating times + (1|experimental No.) + (1|male) + offset(numbers of total events). Experimental No. refers to the laboratory in which the behavioral experiments were performed and individual male and female (only in swapped dyads) identification numbers of males for the fixed test and females for the swapped test were included as random intercepts (e.g., model = latency ~ mating times + type + type : mating times + (1|experimental No.) + (1|male)). I constructed both full models including mating times as an explanatory variable and null models with no explanatory variable, and then compared the models using the likelihood ratio test. To select the model with the best predictability, I compared the Akaike Information Criterion (AIC) between the 2 models. When the likelihood ratio test indicated a significant effect on the mating times ($P < 0.05$), adjusted P values calculated using the emmeans package (version 1.6.1) with the Tukey method are shown for post hoc test.

Results

Mating experience of naïve males decreased the latency to mate with the same partner

To investigate how the mating experience of naïve males could influence mating behavior in medaka fish, I performed mating tests using naïve males and experienced males that had mated with females more than 7 times. To prepare naïve males, I separated juvenile males into groups and bred them without any females until performing the mating tests. I also prepared sexually mature females (>3 months after hatching) for the mating test. The mating tests were carried out using 21 (naïve males) and 23 (experienced males) fixed dyads as a control for 7 days (7 times) (Fig. 1A, 1B). Next, I used experienced males that had obtained the previous mating experience with the same female more than 7 times. To compare the experienced males with the naïve males paired with novel partners, I changed the female partners of the experienced males just before the mating tests. The latency to mate was defined as the interval from "releasing the male" to "wrapping with spawning". To evaluate the transition of the latency to mate between naïve and experienced males, I used a GLMM with a gamma distribution, because the previous study revealed mating latencies can be approximated as gamma distribution[19] and a GLMM can include information of individual differences, [AIC (full) = 1758.40, AIC(null) = 1790.37, deviance = 1736.40, deviance(null) = 1782.37, Chisq = 45.98, Df = 7, Pr(>Chisq) = 8.84E–08]. Results of the post-hoc test revealed that the latency significantly decreased after the mating experience and biased the distribution for 7 days only in naïve males, and not in experienced males (Fig. 2, Table 1). Together, these findings indicated that the male mating experience influenced medaka mating behavior only in naïve males. Therefore, I concluded that the mating experience of naïve males decreased the latency to mate with the same partner.

Mating experience of naïve males enhanced both male and female mating activities.

I further examined which behavioral component could influence the latency to mate in naïve males in repeated mating tests. First, I compared the number of courtship displays [AIC (full) = 840.97, AIC(null) = 871.26, deviance = 820.97, deviance(null) = 865.26, Chisq = 44.29, Df = 7, Pr(>Chisq) = 1.88E–07] and the latency to the first courtship display [AIC (full) = 227.12, AIC (null) =256.26, deviance = 205.12, deviance (null) = 248.26, Chisq = 43.14, Df = 7, Pr(>Chisq) = 3.13E–07] between naïve and experienced males, revealing that the latency to the first courtship display significantly decreased after the mating experience in naïve males (Fig. 3A, Table 2). On the other hand, the number of courtship displays varied widely [AIC (full) = 840.97, AIC (null) = 871.26, deviance = 820.97, deviance (null) = 865.26, Chisq = 44.29, Df = 7, Pr(>Chisq) = 1.88E–07] in either naïve or experienced males (Fig. 3B, Table 4). Next, I compared the latency to mate after the first courtship display, which negatively correlated with the degree of female receptivity [17,20], because females with high receptiveness tend to accept males immediately after the first courtship display. The latency was significantly decreased [AIC (full) = 365.53, AIC (null) = 384.91, deviance = 343.53, deviance (null) = 376.91, Chisq = 33.38, Df = 7, Pr(>Chisq) = 2.25E–05] in naïve males (Fig. 3C, Table 3), and strongly suggested that the female mating experience with the naïve males enhanced female receptivity. Furthermore, I analyzed the number of wrappings [AIC (full) = 440.71, AIC (null) = 433.09, deviance = 420.72, deviance (null) = 427.09, Chisq = 6.37, Df = 7, Pr(>Chisq) = 0.497] and the number of wrapping rejections [AIC (full) = 313.55, AIC (null) = 318.683, deviance = 293.55, deviance (null) = 312.68, Chisq = 19.14, Df = 7, Pr(>Chisq) = 0.00777], and these numbers varied widely in both naïve and experienced males (Fig. 4, Table 5). Next, I analyzed behavioral transition probability in the mating tests, and found no significant differences in any of the behavioral transitions (Fig. 5, Table 8). Accordingly, I revealed that the

mating experience in naïve males shortened “the latency to the first courtship display” as well as “the latency to mate after the first courtship display”, suggesting that the mating experience in naïve males enhanced both male and female mating activities.

Repeated mating experience with the same naïve males shorten the transfer distance of both sexes medaka during the matings

To further confirm whether mating experiences with the naïve males could change female receptivity, I decided to analyze medaka locomotor activities during mating tests. I expected that the total swimming distance of both males and females during mating should increase if female often exhibited rejection behaviors toward males. I analyzed 23 mating videos (11 naïve males and 12 experienced males) which were converted 12 frame per minute to reveal each moving distance. I compared the total swimming distance of male [Fig. 6A, AIC (full) = 519.85, AIC (null) = 526.04, deviance = 507.85, deviance (null) = 520.04, Chisq = 12.19, Df= 3, Pr(>Chisq) = 0.00676] and female [Fig. 6B, AIC (full) = 527.59, AIC (null) = 530.30, deviance = 515.59, deviance (null) = 524.30, Chisq = 8.712, Df= 3, Pr(>Chisq) = 0.0338] during mating behaviors, revealing that the total swimming distance of both male and female significantly decreased after the mating only in naïve males.

Naïve males approached unfamiliar males than familiar ones

As above, I revealed naïve males showed high mating activities only to the first mate partner females, implying naïve males had the social preference to the familiar individuals. Therefore, I hypothesized that naïve males exhibit social affiliation (the tendencies to make cohesive groups) with familiar individuals, because, in some fish species, juvenile fish tends to form groups with familiar/kin individuals[21–23]. To examine whether naïve male medaka exhibit social affiliation toward familiar individuals, I performed a three-chamber test using 11 naïve males. I recorded and

analyzed approaching behaviors of naïve males for 5-min (Fig. 7). The mean of the individual distance between focal males and familiar ones was significantly higher than that of unfamiliar ones (Fig. 8, 9, Paired T test, t = 2.7751, df = 11, P = 0.01806), showing that naïve males have the ability to discriminate between unfamiliar and familiar males in medaka. However, unexpectedly, naïve males preferred to approach unfamiliar males, suggesting naïve males didn't have a social preference to familiar males.

The behavioral change in naïve males occurred only in fixed dyads

The mating experience in naïve males mainly influenced the latency to mate and the latency to the first courtship display in fixed dyads in 7 continuous mating tests. Here I examined whether this effect was specific for fixed dyads. To compare mating behavior between fixed dyads and swapped dyads, I performed mating tests for 3 days (Fig. 10, each n = 8). 'Fixed' dyads were those in which the male mates with the same female every day, while 'swapped' dyads were those in which the male mates with a different female each day. The latency to mate was significantly decreased only in the fixed group [AIC (full) = 453.39, AIC (null) = 468.10, deviance = 437.39, deviance (null) = 462.10, Chisq = 24.71, Df = 5, Pr(>Chisq) = 1.58E–04, Fig. 11, Table 6]. The latency to the first courtship display was also significantly decreased in the fixed group, but did not change in swapped dyads [AIC (full) = 45.48, AIC (null) = 62.57, deviance = 29.48, deviance (null) = 56.57, Chisq = 27.09, Df = 5, Pr(>Chisq) = 5.48E–05, Fig. 12A, Table 7]. The latency to mate after the first courtship display; index of female receptivity [Fig. 12B, AIC (full) = –11.34, AIC(null) = –11.99, deviance = –27.34, deviance (null) = –17.99, Chisq = 9.35, Df = 5, Pr(>Chisq) = 0.0959], numbers of other events (Fig. 13, [courtships (AIC (full) = 177.50, AIC (null) = 170.56, deviance = 163.50, deviance (null) = 166.56, Chisq = 3.06, Df = 5, Pr(>Chisq) = 0.691], [wrapping (AIC (full) = 148.86, AIC (null) = 140.99, deviance = 134.86, deviance (null) = 136.99, Chisq = 2.13,

Df = 5, Pr(>Chisq) = 0.831], [wrapping rejection (AIC (full) = 92.20, AIC (null) = 86.47, deviance = 78.20, deviance (null) = 82.48, Chisq = 4.27, Df = 5, Pr(>Chisq) = 0.511]), and behavioral transitions (Fig. 14, Table 9) did not significantly change in either fixed or swapped dyads. The latency to mate and the latency to the first courtship display significantly were decreased after the second encounter (day 3). These findings strongly suggest that the naïve males recognized the first mating partner and thus the latency to mate was decreased according to the mating experience.

Discussion

The findings of the present study revealed that naïve male medaka fish alter their mating behavior according to their mating experience. Interestingly, the pattern of behavioral alterations after the first mating in medaka fish was quite different from those of other species such as fruit flies [2,24], mice [3,4], and rats [5], in which the mating experience increases male sexual motivation, but does not influence mating activity toward familiar females. In medaka fish, mating experience with the same partners decreased the latency of naïve males to mate, but not experienced males. This finding suggests that naïve medaka males recognize the female that they mated with previously and mating activity with the familiar female is enhanced, while in experienced medaka males, mating activity is not enhanced for the familiar female. In addition, I observed the interesting phenomenon that female receptivity was enhanced only after they mated with naïve males. The mating experience of naïve males significantly decreased "the latency to the first courtship" as well as "the latency to mate after the first courtship. The index of the latency to the first courtship negatively correlated with male mating activity, while the latency to mate after the first courtship negatively correlated with female receptiveness. The previous study showed that females could be visually familiarized with proximally located males and that the visual familiarization enhanced female receptiveness toward the familiarized males [25]. If naïve males were in close proximity to the female more frequently than the experienced males, it could enhance female receptiveness via visual familiarization. Further studies are needed to investigate whether naïve males tend to keep close proximity to the mating partner or not. Furthermore, in this study, I revealed the number of wrapping rejections increased in the fifth mating with experienced males than twice and third matings with naïve males, suggesting female medaka may tend to reject experienced males (Fig.

4B). In addition, the total swimming distances of males and females were significantly decreased with multiple mating experiences in naïve males (Fig. 6A). It is also consistent with our findings that the females have high sexual receptivity to naïve males that were mated with and didn't escape from them (Fig. 6B, Fig. 15).

Why was mating activity with the same partner enhanced for naïve males and not for experienced males? What is the adaptive significance of the formation of the relationship between naïve males and females like a "pair-bonding"? Under normal conditions where males have many chances to encounter females, they do not have to select a specific female as a mating partner. Some possible explanations for the adaptive significance of these behaviors are as follows. One possibility is that under an environmental condition where males have few chances to encounter females, males could change their mating strategy (Fig. 16). Under this condition, males might enhance mating/courtship activity toward a specific female that they had mated once. As a result, female mate preference might be increased by familiarization with the naïve male that had mated. Another possibility is that the mating experience erases a certain behavioral property of juvenile individuals, which can recognize familiar individuals and tend to approach them to form a kin group [21]. Humbug damselfish [22] and three-spot dascyllus [23] juveniles tend to approach familiar individuals. Juvenile guppies tend to form kin groups to achieve effective transfer and protect against predators [21]. Juvenile cichlid fish maintained in a kin group grow faster [26] and approach a predator for surveillance more often than solitary individuals that do not live in a group [27]. In contrast, I revealed that naïve adult males prefer to approach unfamiliar males in this study. This result was inconsistent with the former hypothesis. Therefore, I could propose two reasons for this result. In cichlid fish, sexally-matured males tend to approach unfamiliar males, because they pay more attention to unfamiliar males and turn them out to protect their familiar group[28]. I will have

to check that medaka males have the same behavioral property irrespective of mating experience. The other is that the naïve males that I used in this experiment were too old (>5 months old), because male medaka matures within 2–3 months usually under laboratory conditions. Further studies are needed to elucidate whether juvenile and naïve medaka fish tend to approach familiar mates to form kin groups like other fish species.

Chapter2

The effect of mating experiences on brain gene expressions in naïve male medaka

Introduction

In rodents, the behavioral change triggered by the first mating experience is associated with changes in the neural circuits and substrates in the brain as mentioned above in the section of "General Introduction". These works revealed that the morphological changes in some specific brain regions such as mPOA according to the male first mating experience[4,10,13]. However, little attention has been paid to the impact of the male mating experiences on the whole-brain level. Therefore, I decided to reveal this question using small fish with the small brains because it enables us to analyze gene expression on the whole-brain level. Previous studies revealed that behavioral states correlate gene expression patterns of whole-brain samples in small fish such as zebrafish[29] and cichlid[30]. For example, gene expression patterns of the whole brains differ in social contexts such as a mirror fighting or a real opponent fighting, suggesting cognitions of social partners are related to neurogenomic responses in zebrafish[29]. In addition, Renn et al reported social state varies gene expression patterns of hormone precursors (Somatolactin, Arginine-vasotocin, Prolactin)[30] in cichlid, suggesting behavioral experiments alter hormone levels in the brain.

Therefore, I decided to perform RNA-seq of male medaka samples to reveal the molecular mechanism and candidate hormones triggered by the mating experience in naïve males. Next, I measured the amount of the candidate hormone using naïve and post-naïve male samples.

Materials & Methods

Fish and breeding conditions

Medaka fish (*Oryzias latipes*; drR) were maintained as described (Chapter 1).

RNA-Seq and Data Analysis

Whole brains with the pituitary were collected from naïve males with no mating experience and post-naïve males (after 2 mating experiences) in the morning just before the third mating. The dissected brains were stabilized with RNA-later (Thermo Fisher Scientific, USA) until the extraction steps. Total RNAs were extracted using TRI Reagent (Cosmo Bio, Japan) and then purified using an RNeasy plus mini kit (Qiagen, Germany). RNA extracts with the same concentrations from 3 individuals were pooled together to form 1 RNA-seq sample. There were 2 naïve and 2 post-naïve samples (i.e., 4 total samples). All library preparations and sequencing were outsourced to a company (GENEWIZ, Japan). The cDNA libraries were prepared using a NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA). The libraries were multiplexed and loaded on an Illumina HiSeq X Ten (2 x 150 bp, Illumina, USA). The reads were trimmed using Cutadapt[31] (v4.1) and then mapped to medaka reference genome sequences of Ensembl annotation (ASM223467v1) using HISAT2[32] (v2.2.1). DESeq2[33] (v1.30.1) with the Wald test was used for differential expression analysis. Differentially expressed genes were identified with an FDR adjusted *P*-value < 0.01 and |Fold change| > 2. I used Stringtie[34] (v 2.2.1) to estimate the transcript abundances of each sample and Ballgown[35] (v 2.22.0).

Quantification of thyroid hormone

Firstly, I homogenized male 4 brain samples with 1000 μL 0.9w/w% NaCl and then fractionated 50 μL suspension for the protein quantification. I performed protein quantifications using protein assay Lowry kit (Nakarai tesque, Japan) according to the manufacturer's protocol. Varioskan™ LUX VLBL00D0 (Thermofisher, USA) was used for the absorbance measurement. All quantifications of the thyroid hormone were outsourced to a company (NDTS, Japan). The rest of susceptions were used for the quantification of the thyroid hormone. 10 μL ^{13}C-T3 (Merck, Germany), ^{13}C-T4 (Merck, Germany) for the internal standard and 4 mL Ethanol was added to the rest of susception, and mixtures were centrifuged (12000 rpm, 4˚C, 5 min). The supernatant was dried up and dissolved in 50 μL Ethanol. LC-MS/MS analysis were performed using liquid chromatograph mass spectrometer (Prominence and LCMS-8045, Shimadzu, Japan). Peptides were separated on a column packed with C18 (Cadenza CD-C18, 3.0 mm i. d. x 150 mm, Imtact, Japan) at a flow rate of 0.4 mL/min using the following gradients: 30% buffer B (Methanol) in 0 min, 100% buffer B in 8 min, 100% buffer B in 8 min, 100% buffer B in 10 min, 30% buffer B and 70% buffer A (10 mM, formic acid) in 12.5 min. MS/MS spectra were acquired in ESI (positive) mode.

Results

Mating experience in naïve males changed gene expression in the brain

To evaluate the effect of the mating experience in naïve males on the brain gene expression patterns, I compared gene expression profiles using whole brains between the naïve and post-naïve male medaka that had 2 mating experiences (Fig. 17). In the present study, I performed RNA-seq analysis using the whole brain with the pituitary, because in some fish species such as zebrafish and cichlid, social status has significant effects on gene expression at the whole brain level[29,30]. I identified 20 differentially expressed genes (DEGs) that were upregulated by the mating experience and had a greater than 2-fold change, suggesting that the mating experience could influence brain gene expression in naïve male medaka (Fig. 18, Table 11, 12). I listed the DEGs in descending order of the expression level (FPKM). Interestingly, I found that 3 genes (*tshba*, *dio2*, *klf9*) of DEGs were associated with functional expression of the thyroid hormone system (Table 11). *tshba* encodes a thyroid-stimulating hormone that promotes the synthesis and secretion of inactivated thyroid hormone (T4). *dio2* encodes type II iodothyronine deiodinase, which converts inactivated thyroid hormone to activated thyroid hormone (T3). *klf9* encodes a transcription factor, Krüppel-like factor 9, which is induced by thyroid hormone (T3)[36].

Increase tendency of thyroid hormone after the mating experiences in the male brain

RNA-seq analysis revealed three genes associated with functional expression of thyroid hormone system were up-regulated in post-naïve males than naïve ones. Therefore, I conducted measurements of the thyroid hormone in naïve and post-naïve males brain using LC-MS/MS technique and, I quantified brain protein volume using the Lowry method for the standardization

among each sample (Fig. 19). The amount of the thyroid hormone T3 (T test, t = –0.69602, df = 6.8385, $P = 0.5094$) and T4 (t = 0.132, df = 4.1293, $P = 0.9012$) were no significantly difference between naïve and post-naïve males however, the average amount of T3 was increased 1.17 times in post-naïve samples than naïve samples (Fig. 20).

Discussion

Various neurochemical substrates might be involved in experience-dependent plasticity in social/mating behavior, including oxytocin [37], glutamate [38], and opioids [39]. Among mammals and nonmammalian vertebrates, catecholamines such as dopamine (DA) and norepinephrine (NE) regulate the expression of sexual behaviors [40,41]. The findings of the present study suggest the mating experiences of naïve males induce the upregulation of 3 genes (*tshba*, *dio2*, *klf9*) related to functional expression of the thyroid hormone, and other genes such as *fkbp5 and hapln2*. In rodent brains, early-life stress exposure increases *fkbp5* expression in the brain and FKBP5 regulates glucocorticoid receptor activity [42]. High *fkbp5* expression and early-life stress interact to increase anxiety-like behavior mediated by AKT signaling in association with hippocampal synaptic plasticity [43]. Hapln2 (also called Bral1) is essential for the formation of the functional extracellular matrix and neuronal conductivity in mice [44]. More importantly, transcriptome analysis suggested the possible involvement of thyroid hormone in the effect of experience-dependent plasticity in mating behavior. Thyroid hormone is required for brain maturation and development [45,46] as well as experience-dependent behavioral plasticity in chick imprinting[47]. In newly hatched (1-day-old) chicks, imprinting upregulates *dio2* gene expression, which is required for memory formation associated with imprinting[47]. The thyroid hormone is also involved in behavioral plasticity in mating behavior. In Japanese quail, thyroid hormone is a trigger hormone for the release of gonadotropin-releasing hormone in the brain to mature gonads for reproduction [48]. A relationship between seasonal reproduction and thyroid hormone in the brain is reported across species, including mice [49] and fish [50], among seasonally reproductive animals. In this study, sexually mature males were used for the mating test and there was no change of seasonal information (i.e., water

temperature and day length remained the same) in the laboratory, but medaka do show seasonal reproductivity [51], suggesting that the thyroid hormone system is activated in the brain by the mating experience and not a seasonal change. To my knowledge, there are no reports of thyroid hormone activation triggered by the mating experience in any species. In addition, *klf9,* which is a transcription factor dependent on the thyroid hormone [36], contributes to dendritic spine maturation in the mouse hippocampus [52]. The mating experience shapes mating behavior and sexual motivation in association with morphological changes in the mPOA, the center of male mating behavior in rodents [53,54]. Therefore, the mating experience may shape male mating and social behavior by changing the neuronal morphology according to thyroid hormone levels in male medaka (Fig. 21). The center of the mPOA, including the sexually dimorphic nucleus of the preoptic area, is more activated in naïve male rats than in experienced male rats [13]. In addition, knockdown of *vgf* expression erases the behavioral maturation dependent on the first mating experience[5]. Interestingly, thyroid hormone regulates *vgf* expression in hamsters [55], implying that experience-dependent behavioral changes in male rodents are also related to the thyroid hormone system.

General discussion

In the first chapter, I revealed that the mating experience of naïve males altered the mating behavior of both males and females. Naïve males could discriminate between familiar and unfamiliar females and the mating experiences enhanced high mating activity only toward the former. In addition, females accepted naïve males immediately after several mating experiences, suggesting that females could discriminate between I and experienced males and increase mating receptivity to the former. To my knowledge, this study is the first report to show that mating experiences in naïve males enhanced the mating activity of a specific pair in any species. In prairie voles (*Microtus ochrogaster*), monogamous species, the mating experience enhanced the mating activity of a specific pair, which could lead to the establishment of stable pair-bonds[56]. In medaka fish, the naïve males seemed to form transient pair-boding like prairie voles for only a few days, while experienced males did not have any mating preference toward the previous mating partner and courted immediately toward the novel females. My findings could give us an interesting question "Why does the naïve male form pair-bonding in medaka fish? What is the biological significance of this mating system?" Until now, I do not have the answers to this question. However, I suspected that males could change their mating strategy, when males have few chances to encounter females (Fig. 16). Under this condition, males might enhance mating/courtship activity toward a specific female that they had mated once. In addition, enhanced mating/courtship activity of naïve males could activate female mate preference of the target female by familiarization. Medaka females could be visually familiarized with proximally located males and the visual familiarization enhanced female receptiveness toward the familiarized males[17,25]. Thus, it would be necessary to investigate whether naïve males tend to keep close proximity to a specific mating partner that they had mated with.

Furthermore, I found that the behavioral phenotype of the naïve males is similar to that of oxytocin mutants in medaka. In medaka fish, the oxytocin pathway was required for unrestricted and indiscriminate mating strategy in males. Wild-type males actively displayed courtships irrespective of familiarity, whereas the mutant males of oxytocin or oxytocin receptor genes exhibited courtships less frequently to unfamiliar females in medaka[57]. It would be interesting that oxytocin might be involved in the behavioral alternation after mating experiences in naïve males because, in some rodents, ejaculation induced a burst of oxytocin release from the neurohypophysis[58].

In the second chapter, I showed that the mating experience of naïve males altered the gene expression pattern of the whole brain (including the pituitary). Among the DEGs, I showed that the expression of three genes involved in the thyroid hormone system was up-regulated after several mating experiences in naïve males. I speculate that thyroid hormone system might play an important role in emergence of the ability of individual recognition, as rapid and rigid learning of objects in hatchings requires thyroid hormone system in formation of imprinting learning in chick[47]. The biological significance of imprinting learning is regarded as mother-infant bond formation. Therefore, thyroid hormone triggered by mating experiences in naïve males might be involved in formation of the paired-bonding like relationship in medaka

Interestingly, in rodent brains, the thyroid hormone regulates the expression of oxytocin. The administration of thyroid hormone increases the promoter activity of oxytocin gene in EC cells (P19 embryonal carcinoma cells)[59]. *In vivo* experiment, oral administration of thyroid hormone increases the oxytocin protein in the pituitary and blood in rats [59]. These results suggest the thyroid hormone in the brain could increase the amount of oxytocin. Taken together with my findings, it might be possible that mating experiences in naïve males induced oxytocin release via

thyroid hormone. Unfortunately, I did not detect any significant effect of mating experience on the amount of thyroid hormone in post-naïve male samples. In this work, I didn't analyze samples with multiple time points. Future studies might be needed to investigate if the mating experience could trigger oxytocin and thyroid hormone release in naïve medaka.

In this paragraph, I would like to discuss on how thyroid hormone system could shape neural networks. In rodent brains, the male mating experience shapes spine structures in the brain as mentioned in the introduction of the second chapter[4,10,5,11]. In the present study, I revealed the mating experience up-regulated *klf9* expression. Kruppel-like factor 9 is a transcription factor induced by thyroid hormone, which could mature the hippocampal dendritic spine in mice[60] as mentioned above in chapter 2. Therefore, my findings may imply *klf9* contributes to neural maturation where the mating or recognition brain region in male medaka. Some brain regions are the central part of the expression of male mating behavior. In fish species, Vs-Vp , which is homologous to the bed nucleus of the mammalian stria terminalis (BNST) , the amygdala in rodents, and NPP, which is homologous to the anterior part of the mammalian preoptic area (POA), are essential for male mating behavior[61,62]. In rodents, also the bed nucleus of the stria terminalis[63] and the preoptic area[64] are the central part of male mating behavior expression. These studies suggest brain regions and functions are conserved among vertebrates. Therefore, the first mating experience might contribute to the dendritic spine maturation in BNST or POA in male medaka. Future work will be necessary to reveal this hypothesis.

In the last paragraph, I would like to explain my speculation on how my work could have impact on other fields such as human brain science. Although most human ancestral mating patterns are believed to be polygamous, the pair-bond is a ubiquitous feature of human mating relationships. The human pair-bond might have the advantage of avoidance of sexually transmitted

diseases spreads[65]. On the other hand, the divorce rate has increased and was about 40–50% of all first marriages in North America[66]. In transient romantic relationships, over 90% of university students in the US had experiences of broken hearts[67]. Based on these reports, I think that to form pair-bond formation is important as same as to end relationships with a specific partner, because some people who cannot forget past partner(s) happen to stalk and domestic violent behavior. However, little attention has been paid to reveal the molecular mechanism of these huge social problems. In the present study, I found that the naïve males form transient pair-boding, while experienced males lost any pair-boding of the previous mating partner in medaka. I am expecting that medaka fish might be a good model to study the neural mechanism of how we could change mating strategy (form/break pair-bonding) based on social conditions/contexts. Therefore, I hope that my findings have the potential to be applied to not only biological science but also human psychology and drug discoveries for such a huge social problem of stalking and domestic violence.

Data availability

All sequence read data are available from DDBJ (DRA013480, https://ddbj.nig.ac.jp/resource/sra-submission/DRA013480).

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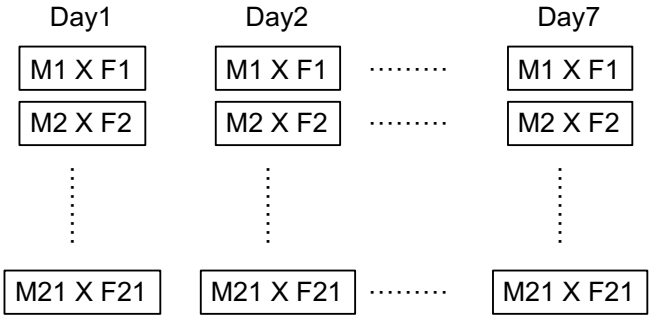
Figures

(A)

Naïve males: Rearing in male groups
Experienced males: > Matings 7 times with the other females

(B)



(C)

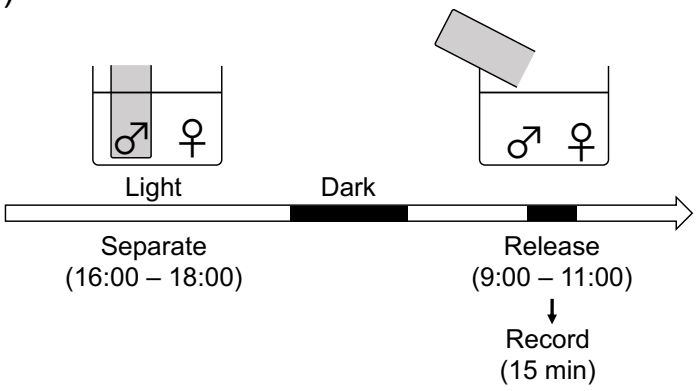


Figure 1 Design of the mating test using naïve males. (A) Information on males that were used in this study. Experienced males were defined as males with mating experiences more than seven times before the mating tests in this study. (B) (C) Outline of the behavioral experiment. (B) Twenty-one (naïve) and 23 (experienced) males were used for the study. Male and female dyads were fixed during the experiment and the experiment was carried out for 7 consecutive days. (C) Experimental procedure. The male was separated a day before the experiment and released to the female the next morning. Their mating behavior was recorded for 15 min and analyzed.

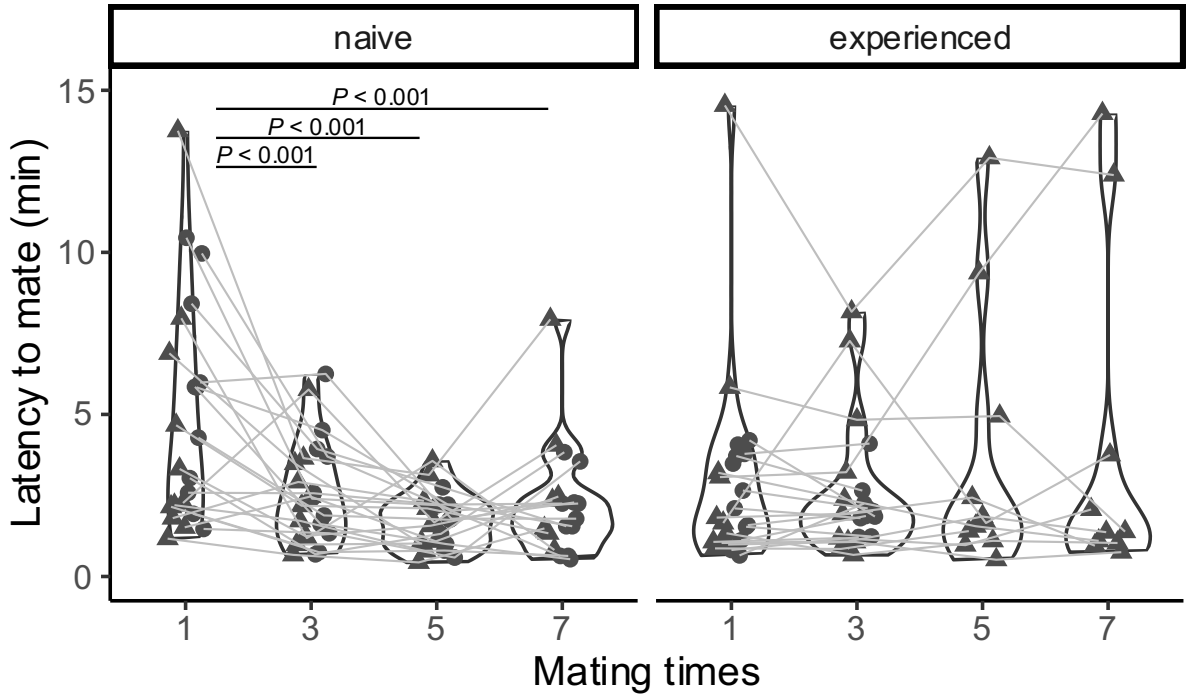


Figure 2 Behavioral test results. Left and right panels show the transition of the latency to mate in naïve and experienced males, respectively. The latency significantly decreased in naïve males depending on the number of matings for 7 days, but not in experienced males. Each dot represents the results of each individual and the shapes show the experimental No (carried out 2 laboratories). *P*-value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model (gamma distribution, log link function).

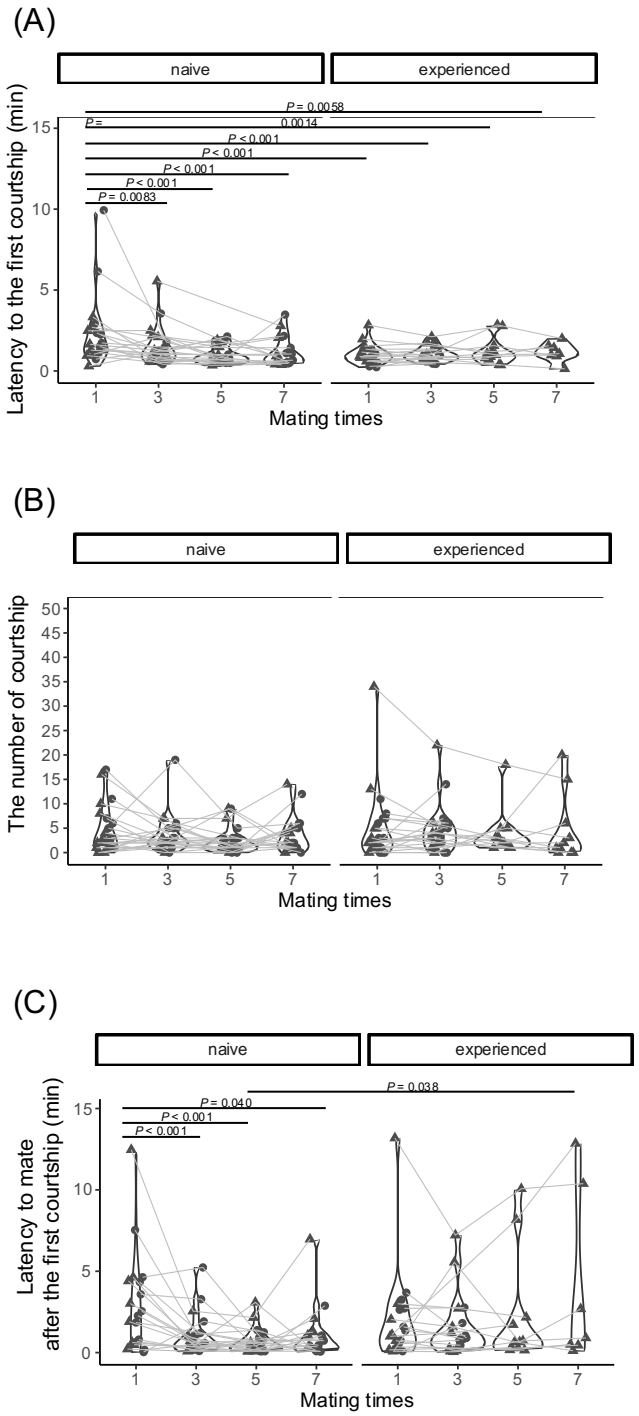


Figure 3 Change in the courtship behavior, latency to the first courtship display (A), and the number of courtship displays (B) and the latency to mate from the first courtship display (C). Each

dot represents the results of each individual and the shapes show the experimental No (carried out 2 laboratories). (B) P-value was abbreviated in the figure because 12 significantly differences were detected. Please see Table S4. *P*-value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model (gamma distribution (A and C) and Poisson distribution (B), log link function).

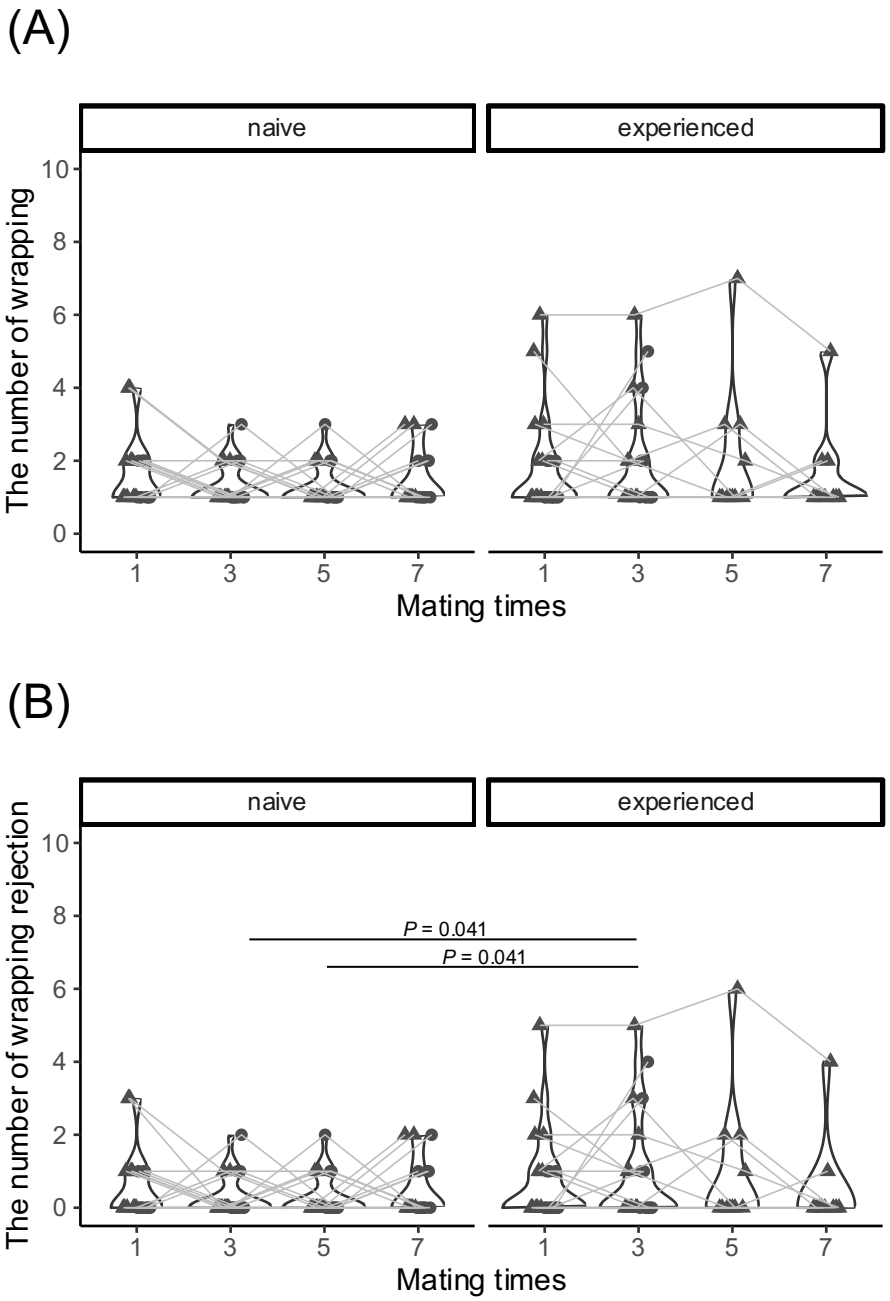


Figure 4 The number of wrappings (A) and wrapping rejections (B). Each dots represents the results of each individual and shapes shows the experimental No (carried out 2 laboratories). *P*-

value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model (poisson distribution, log link function).

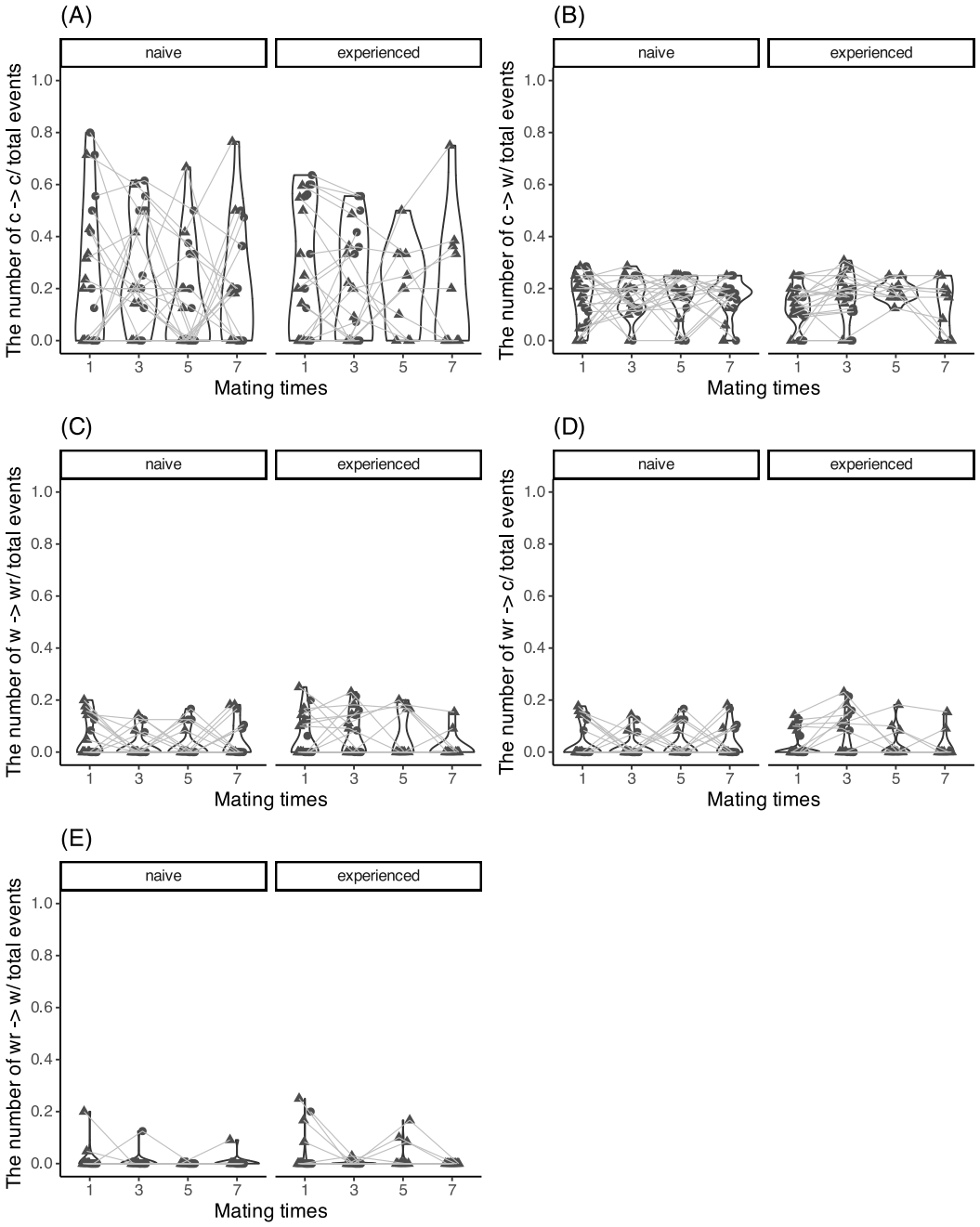


Figure 5 Behavioral transition probability of (A) courtship(c) -> courtship, (B) courtship -> wrapping (w), (C) wrapping -> wrapping rejection (wr), (D) wrapping rejection -> courtship, I wrapping rejection -> wrapping in mating test using swapped dyads. Each dots the results of each

individual and shapes shows the experimental No (carried out 2 laboratories). There were no significantly differences whose *P*-value < 0.05. *P*-value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model [Poisson distribution, log link function, offset(numbers of total events)].

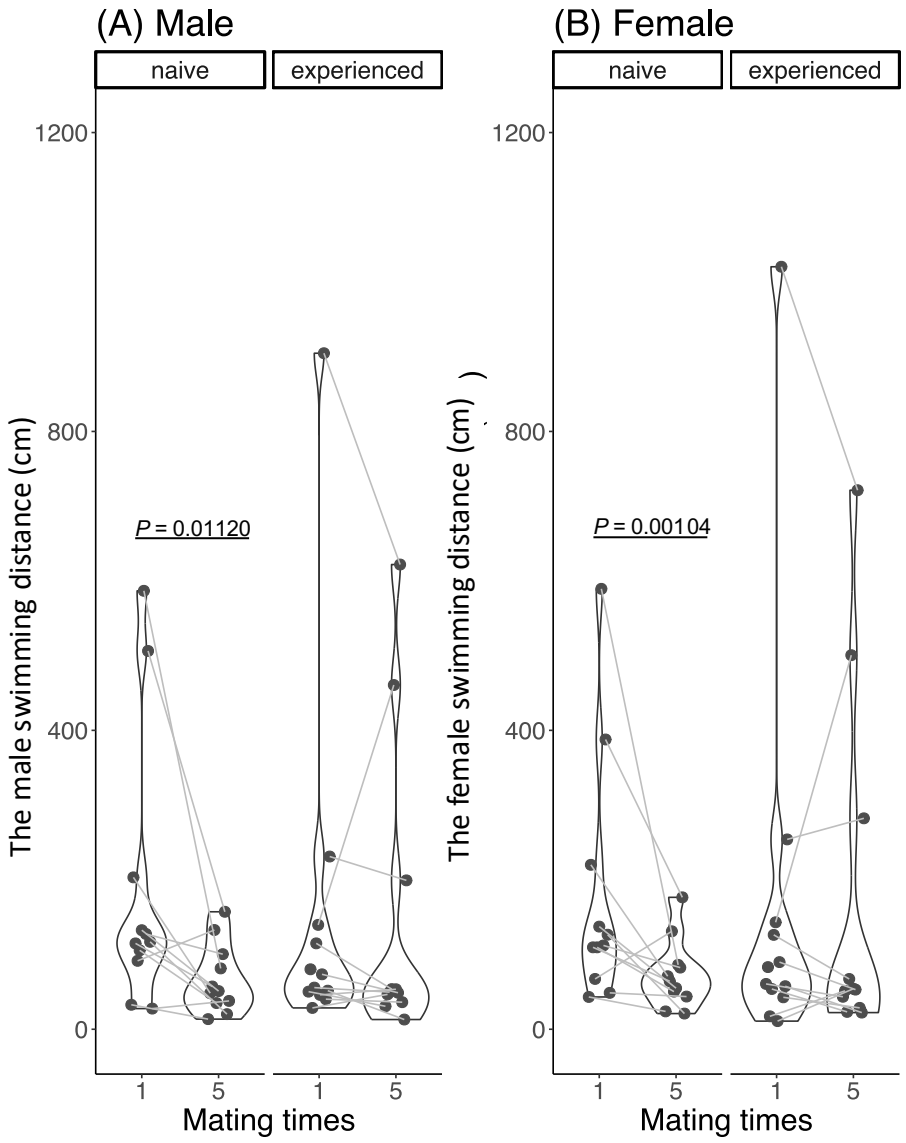


Figure 6 Change in swimming distance of (A) males and (B) females with multiple matings. Each dots the results of each individual. Males and females swimming distances significantly decreased with multiple mating experiences with only same naïve males not experienced ones. *P*-value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model (gamma distribution, log link function).

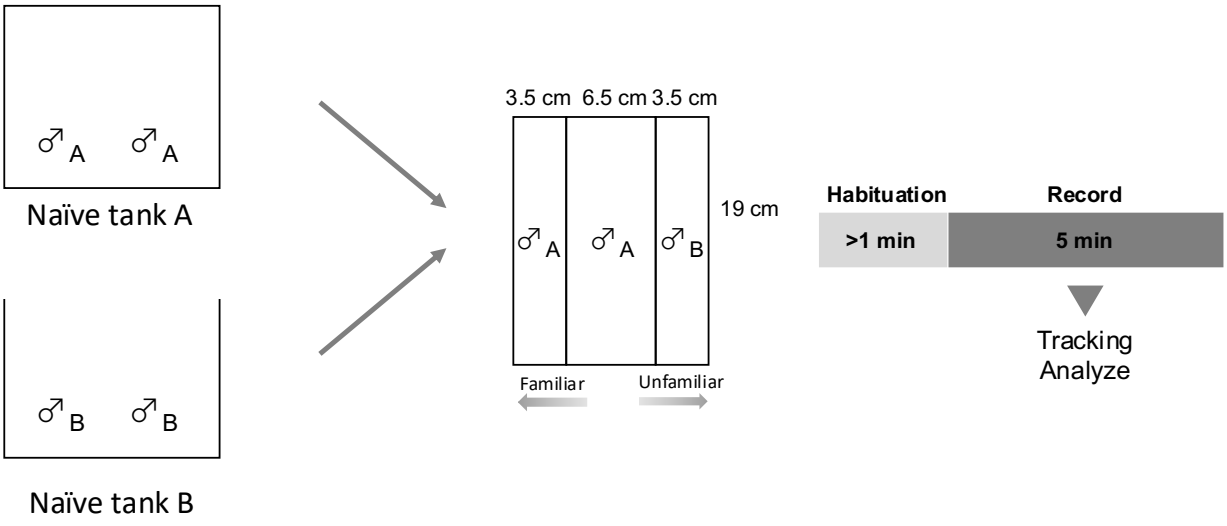


Figure 7 Design of the three-chamber test. I separated naïve males into two groups to generate unfamiliar naïve males. Then, I transferred naïve males (two familiar males and one unfamiliar male) to a three-chamber aquarium and recorded it for 5-min after a habituation than 1-min. I performed individual tracking for each males and calculated individual distances to the familiar or unfamiliar male (each n = 12).

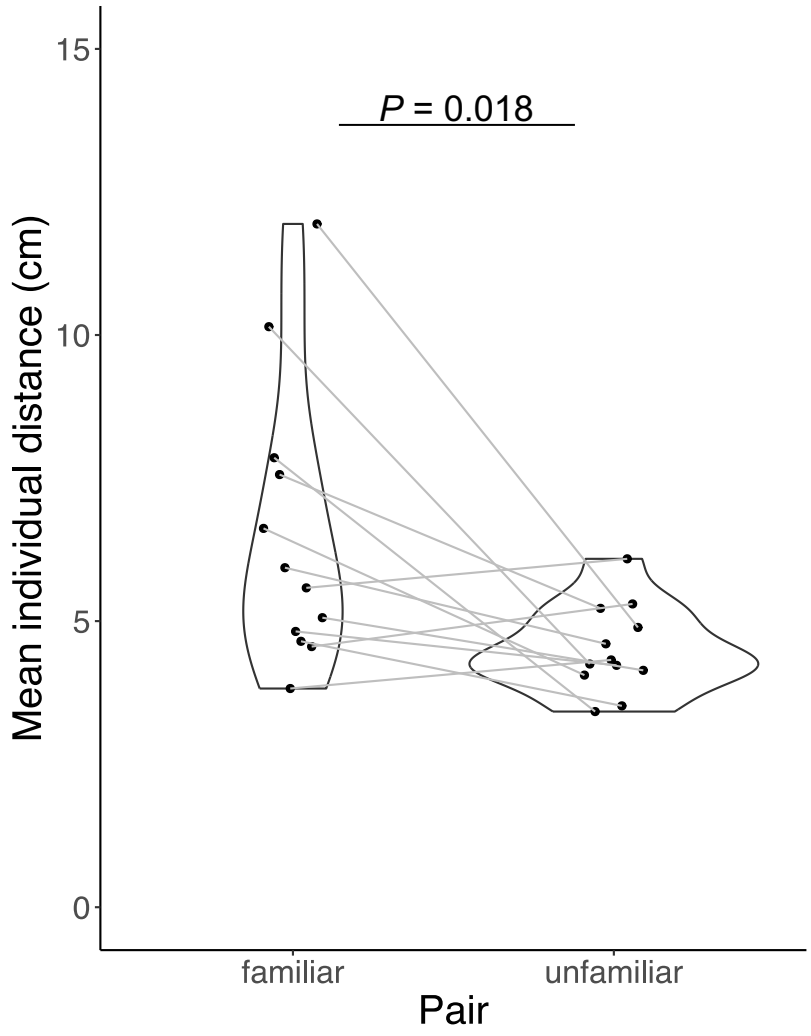


Figure 8 Results of the three-chamber test. Each dots the results of each individual. Males significantly approached to the unfamiliar males than familiar ones. *P*-value shows the results of the paired T test.

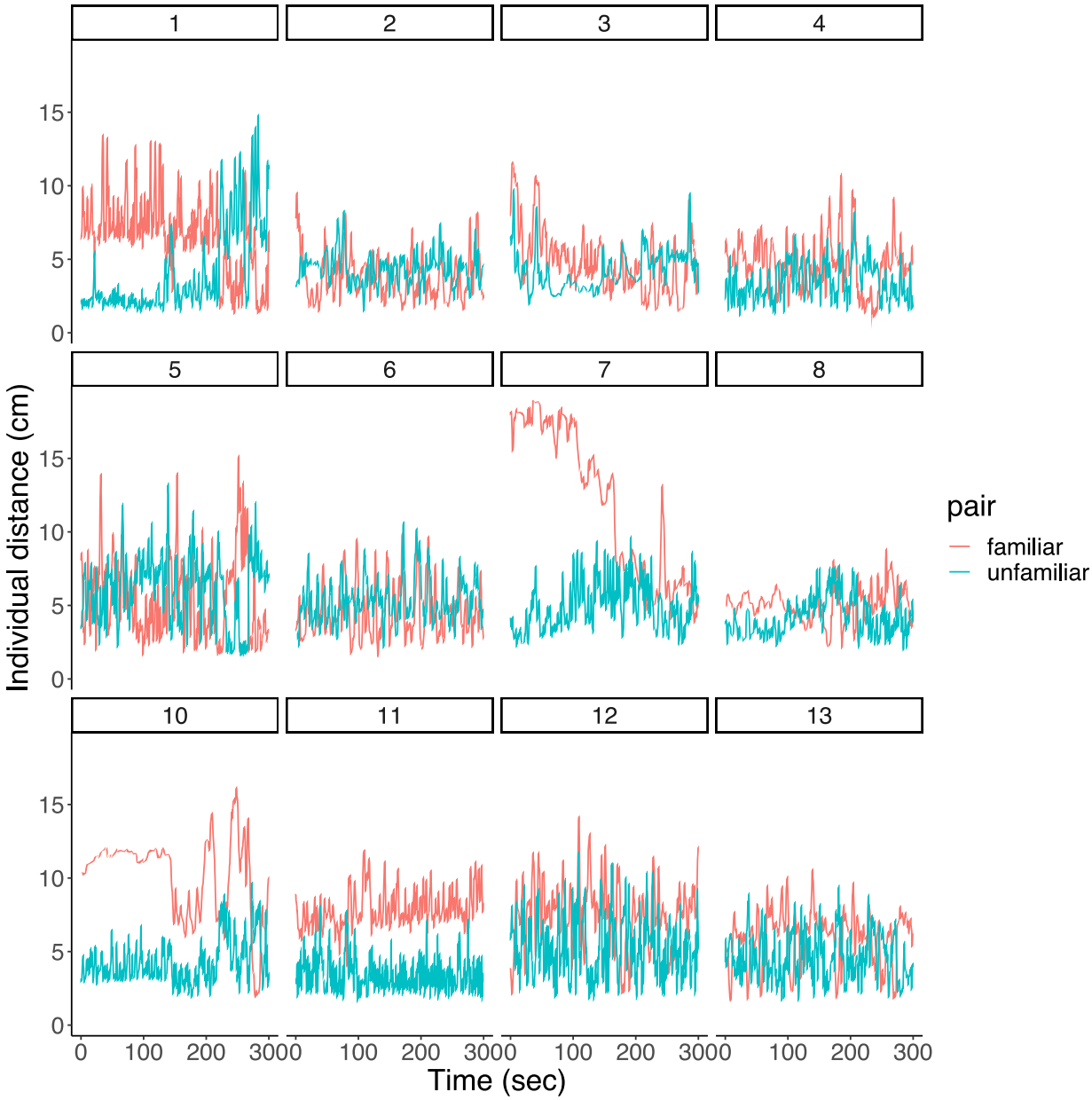


Figure 9 Individual distances of each three-chamber test. Each graph shows relationship between time and individual distance.

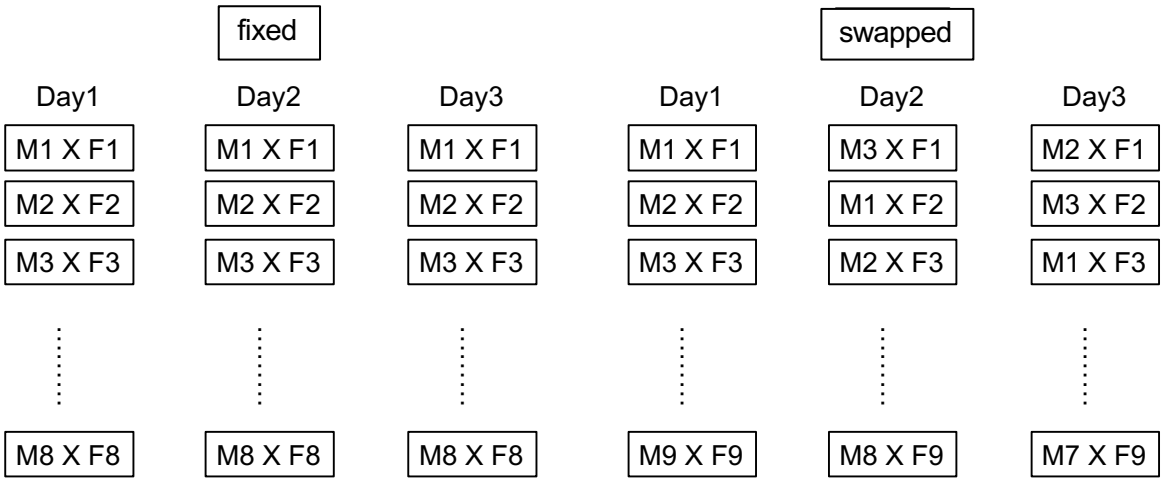


Figure 10 Outline of the behavioral experiment of the swapped mating experiment. Eight (fixed condition) and 9 (swapped condition) males were used for this study.

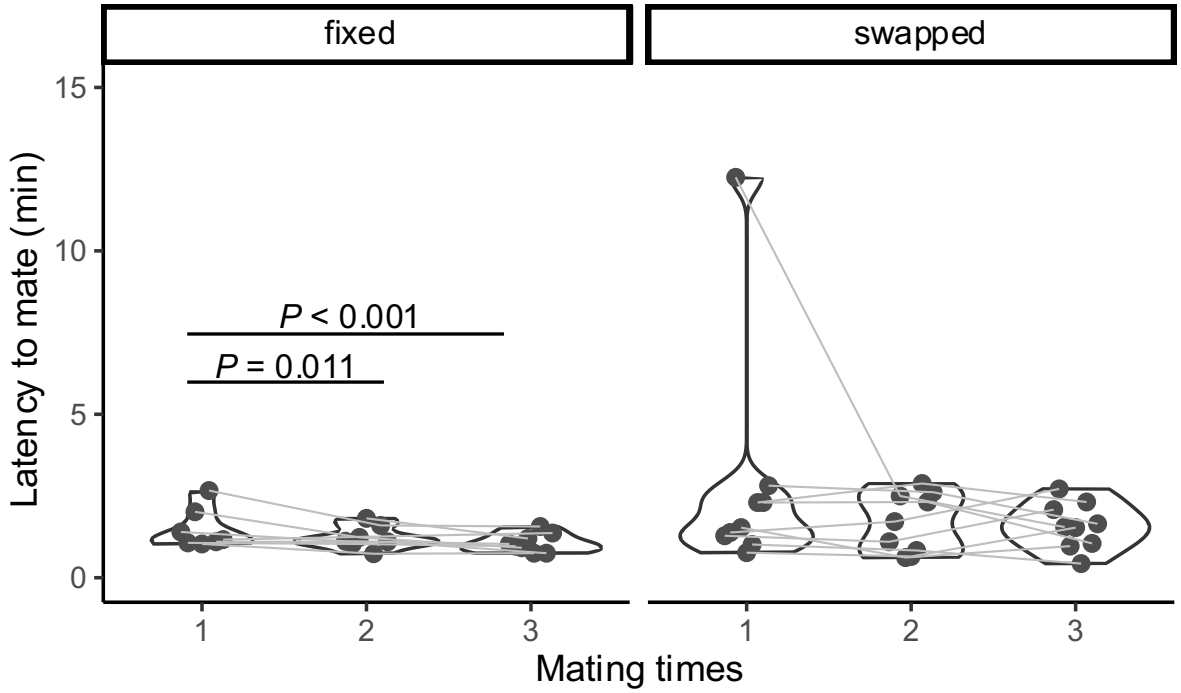


Figure 11 Effect of the familiarization (repeated matings with the same partner) on mating behavior of naïve males. Latency to mate (left: fixed group, right: swapped group). The latency to mate significantly decreased only in the fixed group, and not in the swapped group. Each dot represents the results of each individual and the shapes show the experimental No. *P*-value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model (gamma distribution, log link function).

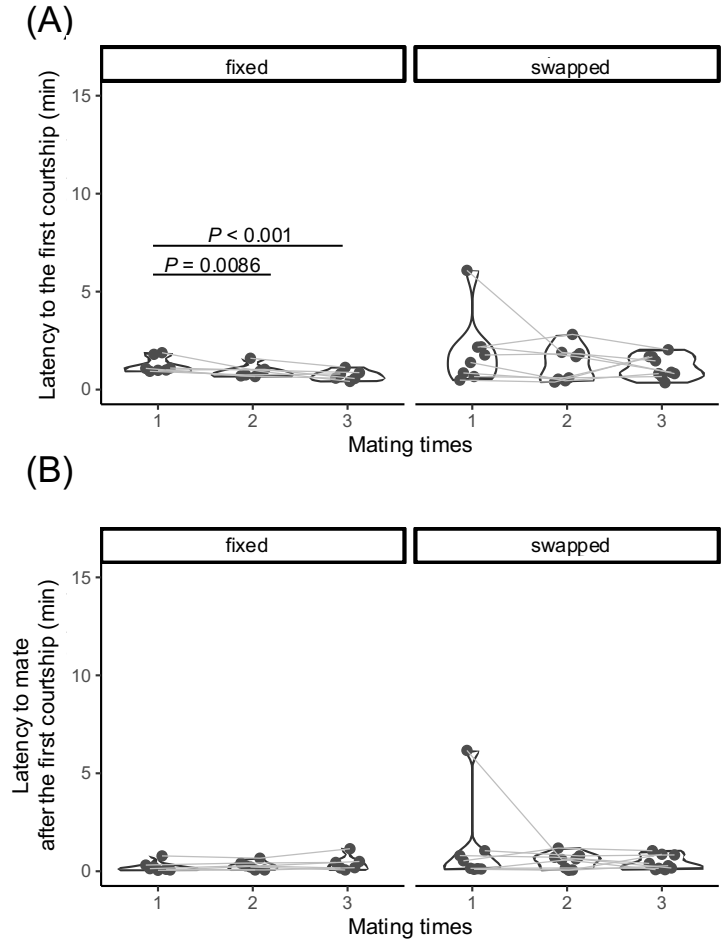


Figure 12 Effect of the familiarization (repeated matings with the same partner) on mating behavior of naïve males. (A) Latency to the first courtship display (left: fixed group, right: swapped group). The latency significantly decreased only in the fixed group, and not in the swapped group. (B) Latency to mate from the first courtship. Each dot represents the results of each individual and the shapes show the experimental No. *P*-value shows the results of the post hoc test with Tukey's adjustment method in a generalized linear mixed model (gamma distribution, log link function).

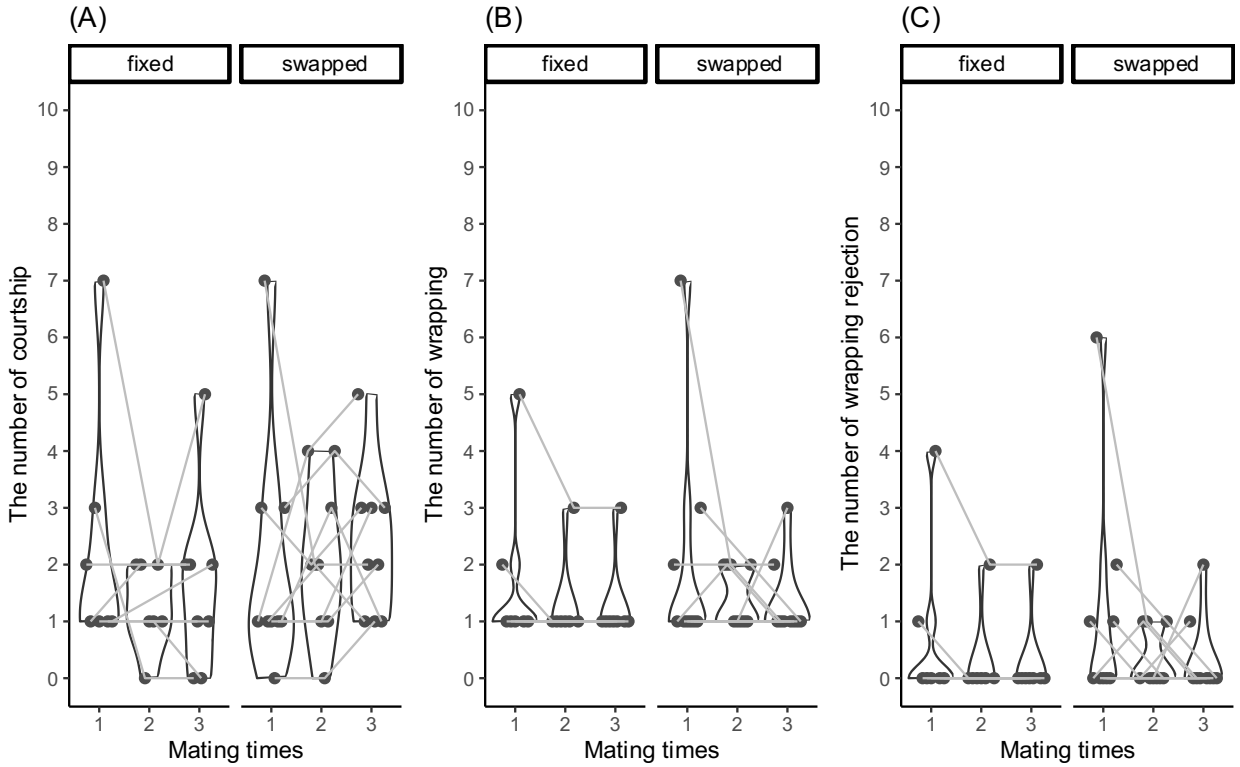


Figure 13 The number of courtship (A), wrapping (B) and wrapping rejection (C) in mating test using swapped dyads. Each dots the results of each. There were no significantly differences whose P-value < 0.05. The way of statistical analysis is as same as in Fig. 4.

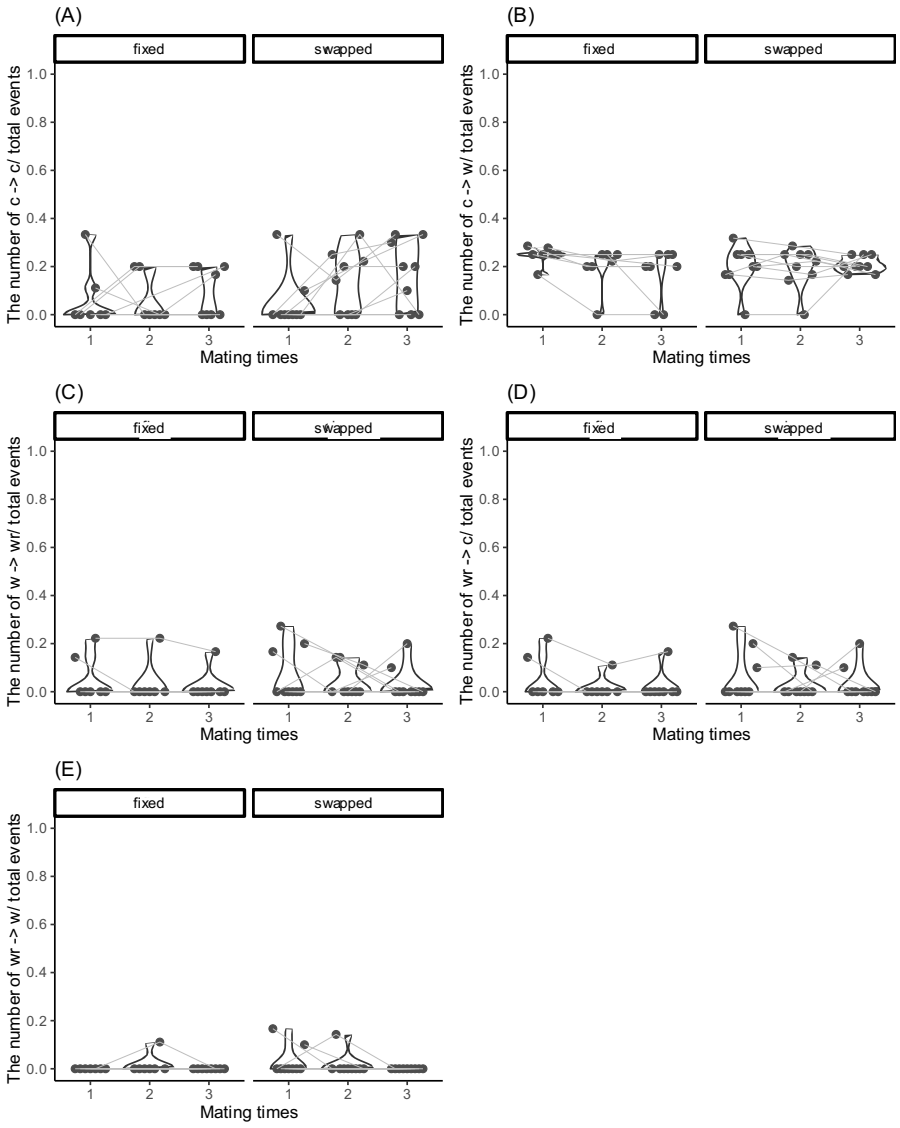


Figure 14 The number of behavioral transition, (A) courtship (c) -> courtship (c), (B) courtship(c) -> wrapping (w) , (C) wrapping rejection (W.R) -> courtship, (D) wrapping -> wrapping rejection, I wrapping rejection -> wrapping in mating test using swapped dyads. Each dots the results of each individual and shapes shows the experimental No. There were no significantly differences whose P-value < 0.05. The way of statistical analysis is as same as in Fig. 5.

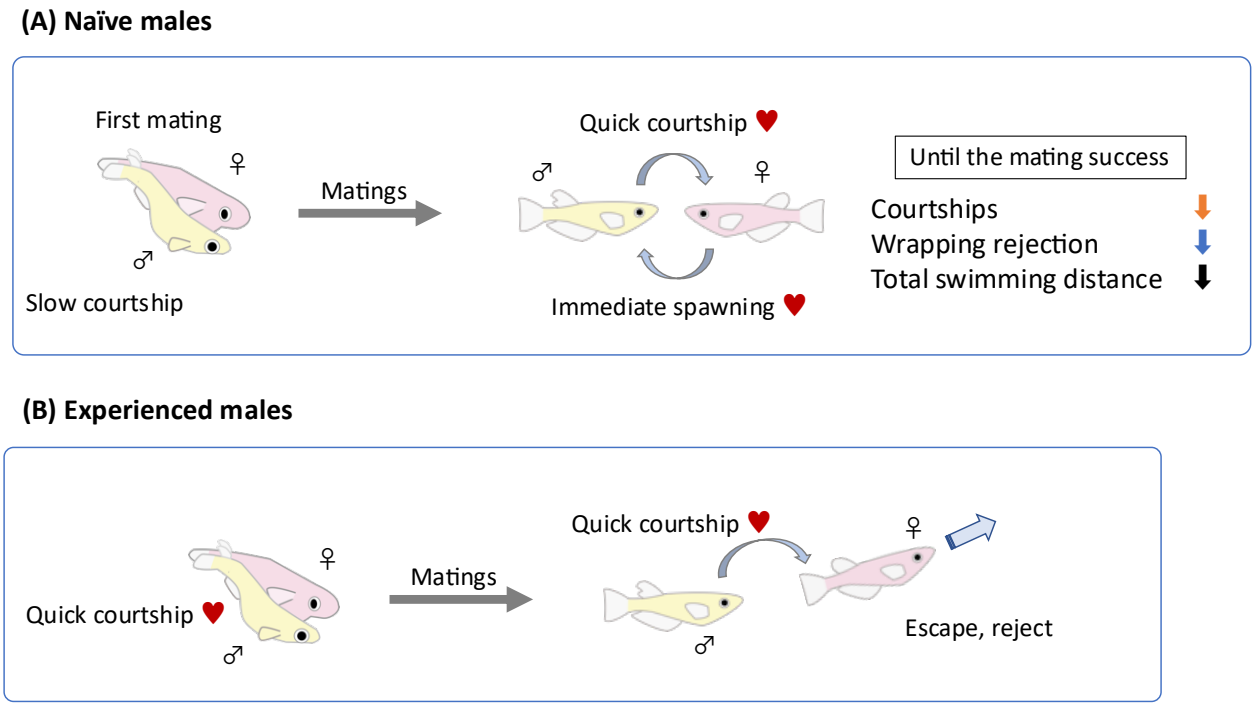


Figure 15 Summary of behavioral phenotypes based on male mating experiences in medaka. (A) Naïve males increased courtship activity according to mating experiences. Mating experiences decreased numbers of courtships, wrapping rejections, and total swimming distance until the mating success. In addition, mating with the same naïve males increased female sexual receptivity. (B) Mating experiences with the same partners didn't change experienced males and females.

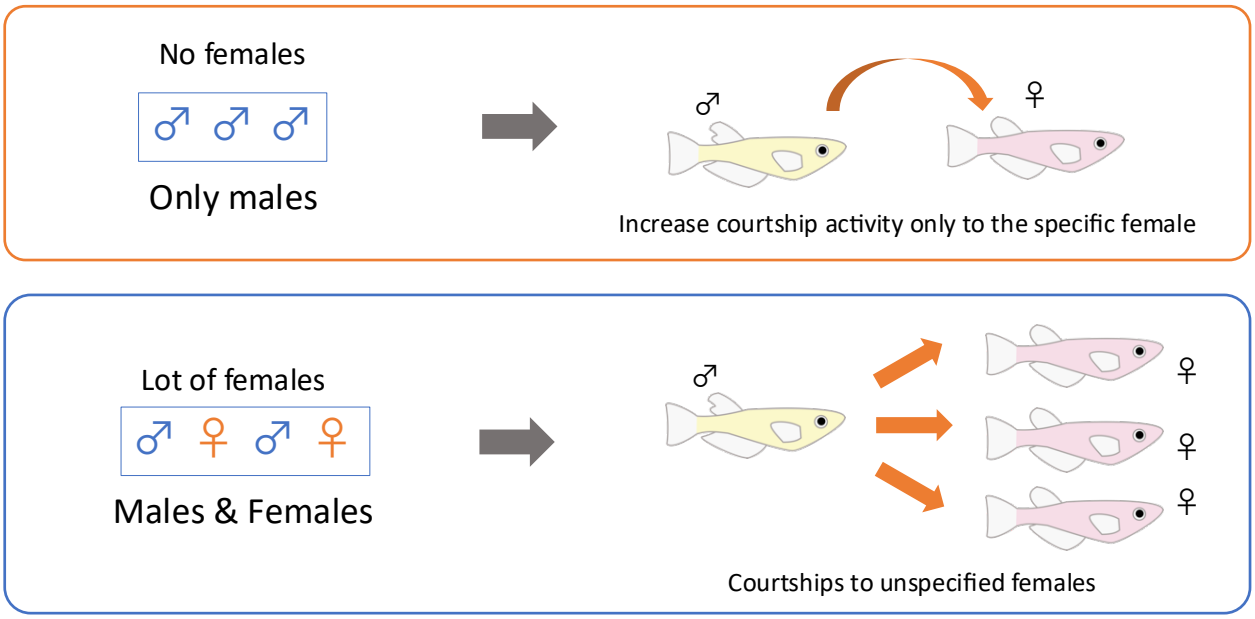


Figure 16 Possible model of the male mating strategy change dependent on the social environment. Naive males were isolated from sexually mature females, while experienced males were with sexually mature females before the mating tests. Therefore, social environmental differences, whether there were females or not, might change the male mating strategy.

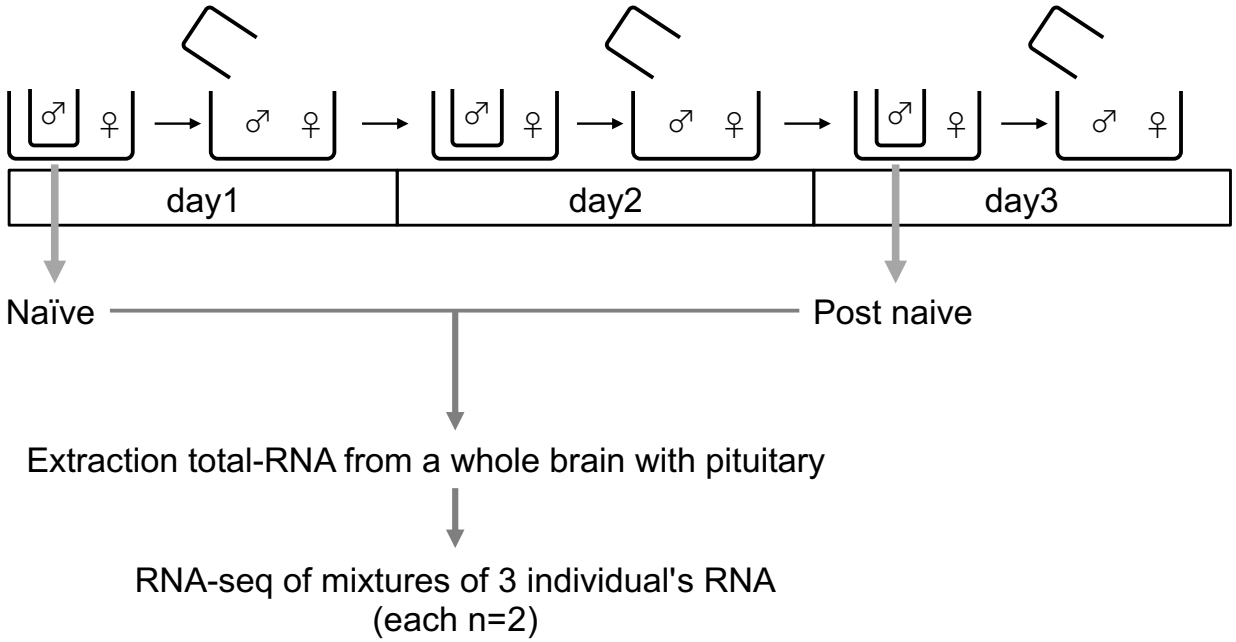


Figure 17 The time course of the extraction brain tissues and outline of the RNA-seq.

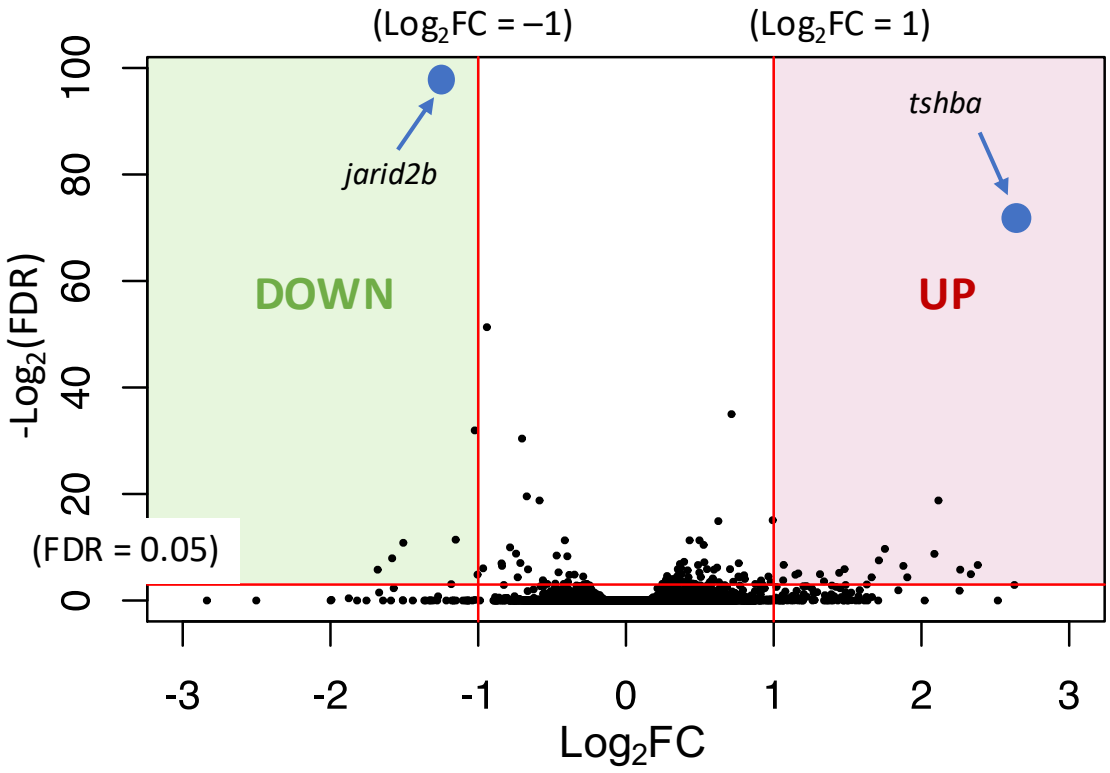


Figure 18 Volcano plots of up-regulated/down-regulated genes in I and post-naïve males. Log_2|foldchange| of gene transcription levels in post-naïve males compared with those in I males.

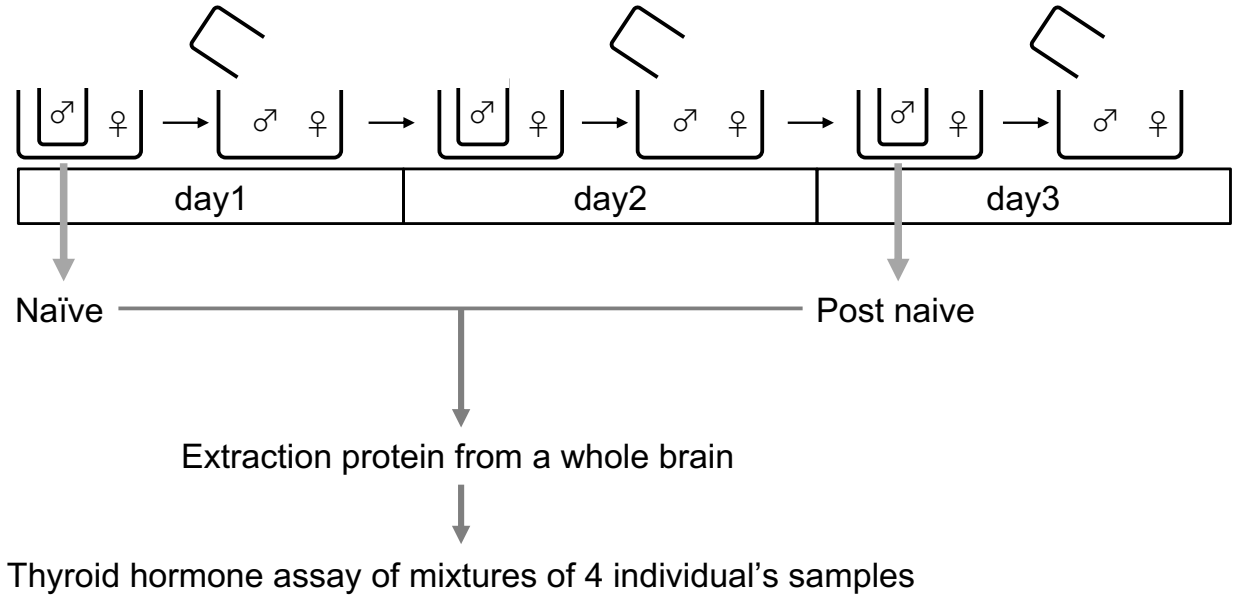


Figure 19 The time course of the extraction brain tissues and outline of the thyroid hormone assay.

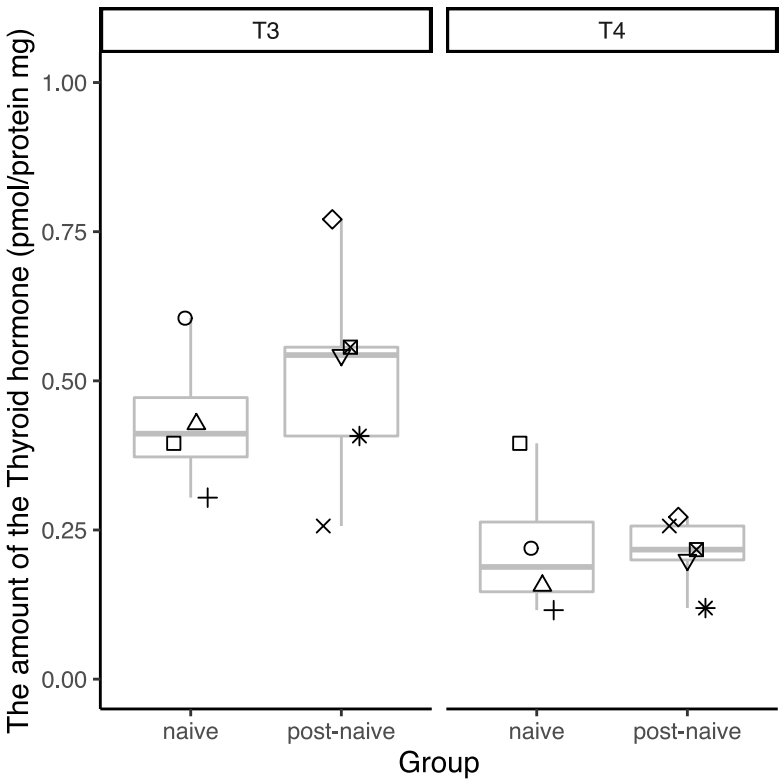


Figure 20 The result of the thyroid hormone assay. Shapes of each dot represents the pooled samples. There were no significant differences (unpaired T-test). The boxplot indicates median (the center line), minimum, maximum, first quartile, third quartile and outlier points.

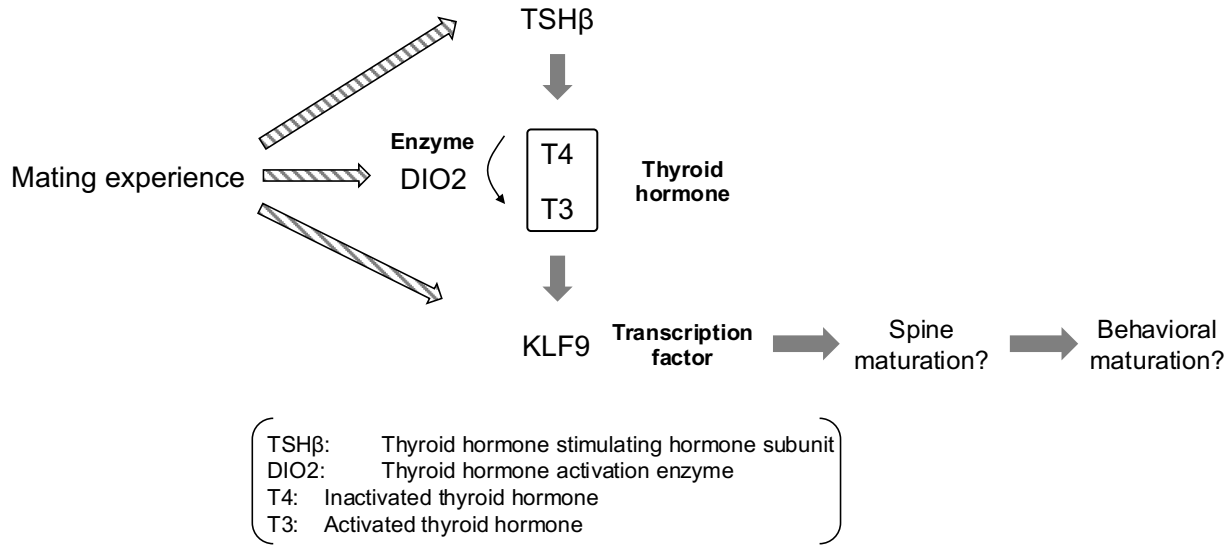


Figure 21 Possible model of the effect of the first mating experience in male medaka. In this study, I found that 3 genes (*tshba*, *dio2*, *klf9*) were upregulated after the first mating experience. *tshba* encodes thyroid stimulating hormone, which activates inactivated thyroid hormone (T4). DIO2 is an enzyme that converts T4 to activated thyroid hormone T3. KLF9 is expressed depending on the T3 level and contributes to spine maturation. The mating experiences may lead to maturation of male mating behavior via neural maturation depending on the thyroid hormone system in the naïve male medaka brain.

Tables

Table 1 The result of the multiple comparison between latency to mate, mating times and types in the fixed group related to Fig. 2.

contrast	estimate	SE	df	z.ratio	p.value
1 naive - 3 naive	0.690	0.151	Inf	4.576	<0.001
1 naive - 5 naive	1.032	0.150	Inf	6.897	<0.001
1 naive - 7 naive	0.724	0.163	Inf	4.432	<0.001
1 naive - 1 experienced	0.523	0.199	Inf	2.631	0.14490
1 naive - 3 experienced	0.518	0.201	Inf	2.574	0.16552
1 naive - 5 experienced	0.461	0.230	Inf	2.001	0.48111
1 naive - 7 experienced	0.462	0.238	Inf	1.943	0.52067
3 naive - 5 naive	0.341	0.151	Inf	2.264	0.31358
3 naive - 7 naive	0.033	0.162	Inf	0.207	1.00000
3 naive - 1 experienced	-0.167	0.196	Inf	-0.856	0.98973
3 naive - 3 experienced	-0.173	0.198	Inf	-0.873	0.98845
3 naive - 5 experienced	-0.230	0.227	Inf	-1.012	0.97288
3 naive - 7 experienced	-0.228	0.235	Inf	-0.971	0.97853
5 naive - 7 naive	-0.308	0.161	Inf	-1.910	0.54402
5 naive - 1 experienced	-0.508	0.195	Inf	-2.610	0.15217
5 naive - 3 experienced	-0.514	0.197	Inf	-2.609	0.15234
5 naive - 5 experienced	-0.571	0.226	Inf	-2.521	0.18659
5 naive - 7 experienced	-0.569	0.234	Inf	-2.433	0.22525
7 naive - 1 experienced	-0.201	0.205	Inf	-0.979	0.97740
7 naive - 3 experienced	-0.206	0.207	Inf	-0.994	0.97536
7 naive - 5 experienced	-0.263	0.236	Inf	-1.116	0.95351
7 naive - 7 experienced	-0.262	0.244	Inf	-1.072	0.96253
1 experienced - 3 experienced	-0.006	0.154	Inf	-0.036	1.00000
1 experienced - 5 experienced	-0.062	0.194	Inf	-0.321	0.99998
1 experienced - 7 experienced	-0.061	0.205	Inf	-0.298	0.99999
3 experienced - 5 experienced	-0.057	0.196	Inf	-0.289	0.99999
3 experienced - 7 experienced	-0.055	0.205	Inf	-0.270	0.99999
5 experienced - 7 experienced	0.001	0.220	Inf	0.006	1.00000

Table 2 The result of the multiple comparison between latency to the first courtship, mating times and types in the fixed group related to Fig. 3A.

contrast	estimate	SE	df	z.ratio	p.value
1 naive - 3 naive	0.481	0.134	Inf	3.580	0.00827
1 naive - 5 naive	0.817	0.135	Inf	6.049	<0.001
1 naive - 7 naive	0.711	0.143	Inf	4.974	<0.001
1 naive - 1 experienced	0.837	0.179	Inf	4.687	<0.001
1 naive - 3 experienced	0.758	0.181	Inf	4.189	<0.001
1 naive - 5 experienced	0.795	0.197	Inf	4.036	0.00140
1 naive - 7 experienced	0.833	0.227	Inf	3.675	0.00581
3 naive - 5 naive	0.336	0.132	Inf	2.541	0.17823
3 naive - 7 naive	0.230	0.144	Inf	1.602	0.74932
3 naive - 1 experienced	0.356	0.170	Inf	2.089	0.42185
3 naive - 3 experienced	0.278	0.174	Inf	1.600	0.75076
3 naive - 5 experienced	0.314	0.188	Inf	1.667	0.70868
3 naive - 7 experienced	0.352	0.219	Inf	1.607	0.74631
5 naive - 7 naive	-0.106	0.143	Inf	-0.742	0.99570
5 naive - 1 experienced	0.020	0.175	Inf	0.113	1.00000
5 naive - 3 experienced	-0.059	0.177	Inf	-0.332	0.99998
5 naive - 5 experienced	-0.023	0.192	Inf	-0.117	1.00000
5 naive - 7 experienced	0.016	0.222	Inf	0.070	1.00000
7 naive - 1 experienced	0.126	0.183	Inf	0.689	0.99729
7 naive - 3 experienced	0.048	0.186	Inf	0.256	1.00000
7 naive - 5 experienced	0.084	0.201	Inf	0.418	0.99990
7 naive - 7 experienced	0.122	0.230	Inf	0.530	0.99951
1 experienced - 3 experienced	-0.079	0.139	Inf	-0.565	0.99925
1 experienced - 5 experienced	-0.042	0.163	Inf	-0.259	1.00000
1 experienced - 7 experienced	-0.004	0.192	Inf	-0.022	1.00000
3 experienced - 5 experienced	0.036	0.167	Inf	0.217	1.00000
3 experienced - 7 experienced	0.074	0.198	Inf	0.375	0.99995
5 experienced - 7 experienced	0.038	0.202	Inf	0.189	1.00000

Table 3 The result of the multiple comparison between latency to mate after the first courtship, mating times and types in the fixed group related to Fig. 3C.

contrast	estimate	SE	df	z.ratio	p.value
1 naive - 3 naive	1.210	0.284	Inf	4.264	<0.001
1 naive - 5 naive	1.520	0.291	Inf	5.222	<0.001
1 naive - 7 naive	0.985	0.317	Inf	3.108	0.03973
1 naive - 1 experienced	0.517	0.371	Inf	1.397	0.85923
1 naive - 3 experienced	0.546	0.374	Inf	1.459	0.82911
1 naive - 5 experienced	0.561	0.417	Inf	1.346	0.88122
1 naive - 7 experienced	0.045	0.473	Inf	0.094	1.00000
3 naive - 5 naive	0.310	0.294	Inf	1.054	0.96596
3 naive - 7 naive	-0.225	0.315	Inf	-0.713	0.99664
3 naive - 1 experienced	-0.692	0.369	Inf	-1.878	0.56627
3 naive - 3 experienced	-0.664	0.374	Inf	-1.775	0.63700
3 naive - 5 experienced	-0.649	0.417	Inf	-1.556	0.77654
3 naive - 7 experienced	-1.165	0.473	Inf	-2.462	0.21210
5 naive - 7 naive	-0.535	0.321	Inf	-1.663	0.71101
5 naive - 1 experienced	-1.002	0.370	Inf	-2.712	0.11870
5 naive - 3 experienced	-0.974	0.372	Inf	-2.615	0.15049
5 naive - 5 experienced	-0.959	0.416	Inf	-2.304	0.29117
5 naive - 7 experienced	-1.475	0.472	Inf	-3.126	0.03763
7 naive - 1 experienced	-0.468	0.379	Inf	-1.233	0.92215
7 naive - 3 experienced	-0.439	0.383	Inf	-1.147	0.94628
7 naive - 5 experienced	-0.424	0.424	Inf	-1.001	0.97445
7 naive - 7 experienced	-0.940	0.481	Inf	-1.956	0.51230
1 experienced - 3 experienced	0.029	0.303	Inf	0.095	1.00000
1 experienced - 5 experienced	0.044	0.354	Inf	0.123	1.00000
1 experienced - 7 experienced	-0.473	0.421	Inf	-1.124	0.95172
3 experienced - 5 experienced	0.015	0.358	Inf	0.042	1.00000
3 experienced - 7 experienced	-0.501	0.417	Inf	-1.203	0.93125
5 experienced - 7 experienced	-0.517	0.417	Inf	-1.237	0.92084

Table 4 The result of the multiple comparison between the number of courtships, mating times and types in the fixed group related to Fig. 3B.

contrast	estimate	SE	df	z.ratio	p.value
1 naive - 3 naive	0.243	0.148	Inf	1.646	0.72227
1 naive - 5 naive	0.600	0.164	Inf	3.648	0.00644
1 naive - 7 naive	0.200	0.156	Inf	1.278	0.90721
1 naive - 1 experienced	-0.651	0.200	Inf	-3.249	0.02561
1 naive - 3 experienced	-0.618	0.204	Inf	-3.035	0.04941
1 naive - 5 experienced	-0.395	0.227	Inf	-1.738	0.66206
1 naive - 7 experienced	-0.519	0.223	Inf	-2.329	0.27750
3 naive - 5 naive	0.357	0.172	Inf	2.071	0.43362
3 naive - 7 naive	-0.043	0.165	Inf	-0.261	1.00000
3 naive - 1 experienced	-0.893	0.207	Inf	-4.322	<0.001
3 naive - 3 experienced	-0.861	0.210	Inf	-4.100	0.00108
3 naive - 5 experienced	-0.638	0.233	Inf	-2.738	0.11138
3 naive - 7 experienced	-0.762	0.229	Inf	-3.333	0.01947
5 naive - 7 naive	-0.400	0.180	Inf	-2.223	0.33763
5 naive - 1 experienced	-1.250	0.219	Inf	-5.708	<0.001
5 naive - 3 experienced	-1.218	0.222	Inf	-5.482	<0.001
5 naive - 5 experienced	-0.995	0.244	Inf	-4.076	0.00119
5 naive - 7 experienced	-1.118	0.240	Inf	-4.665	<0.001
7 naive - 1 experienced	-0.850	0.221	Inf	-3.847	0.00301
7 naive - 3 experienced	-0.818	0.224	Inf	-3.647	0.00647
7 naive - 5 experienced	-0.595	0.246	Inf	-2.415	0.23407
7 naive - 7 experienced	-0.719	0.242	Inf	-2.967	0.06012
1 experienced - 3 experienced	0.032	0.140	Inf	0.231	1.00000
1 experienced - 5 experienced	0.255	0.187	Inf	1.366	0.87263
1 experienced - 7 experienced	0.132	0.183	Inf	0.722	0.99638
3 experienced - 5 experienced	0.223	0.189	Inf	1.183	0.93709
3 experienced - 7 experienced	0.099	0.184	Inf	0.539	0.99945
5 experienced - 7 experienced	-0.124	0.207	Inf	-0.596	0.99893

Table 5 The result of the multiple comparison between the number of wrapping rejections, mating times and types in the fixed group related to Fig. 4B.

contrast	estimate	SE	df	z.ratio	p.value
1 naive - 3 naive	0.693	0.500	Inf	1.386	0.86402
1 naive - 5 naive	0.693	0.500	Inf	1.386	0.86404
1 naive - 7 naive	-0.050	0.450	Inf	-0.110	1.00000
1 naive - 1 experienced	-0.575	0.426	Inf	-1.350	0.87934
1 naive - 3 experienced	-0.866	0.412	Inf	-2.099	0.41551
1 naive - 5 experienced	-0.699	0.461	Inf	-1.518	0.79798
1 naive - 7 experienced	0.057	0.567	Inf	0.101	1.00000
3 naive - 5 naive	0.000	0.577	Inf	0.000	1.00000
3 naive - 7 naive	-0.742	0.534	Inf	-1.390	0.86241
3 naive - 1 experienced	-1.268	0.514	Inf	-2.465	0.21063
3 naive - 3 experienced	-1.559	0.503	Inf	-3.096	0.04118
3 naive - 5 experienced	-1.392	0.544	Inf	-2.561	0.17035
3 naive - 7 experienced	-0.636	0.636	Inf	-1.000	0.97458
5 naive - 7 naive	-0.742	0.534	Inf	-1.389	0.86243
5 naive - 1 experienced	-1.268	0.514	Inf	-2.465	0.21065
5 naive - 3 experienced	-1.559	0.503	Inf	-3.096	0.04118
5 naive - 5 experienced	-1.392	0.544	Inf	-2.561	0.17036
5 naive - 7 experienced	-0.636	0.636	Inf	-1.000	0.97459
7 naive - 1 experienced	-0.525	0.475	Inf	-1.106	0.95577
7 naive - 3 experienced	-0.816	0.464	Inf	-1.759	0.64793
7 naive - 5 experienced	-0.650	0.511	Inf	-1.271	0.90967
7 naive - 7 experienced	0.107	0.609	Inf	0.175	1.00000
1 experienced - 3 experienced	-0.291	0.329	Inf	-0.884	0.98756
1 experienced - 5 experienced	-0.124	0.402	Inf	-0.310	0.99999
1 experienced - 7 experienced	0.632	0.521	Inf	1.213	0.92842
3 experienced - 5 experienced	0.166	0.385	Inf	0.432	0.99987
3 experienced - 7 experienced	0.923	0.508	Inf	1.815	0.60983
5 experienced - 7 experienced	0.756	0.540	Inf	1.401	0.85724

Table 6 The result of the multiple comparison between latency to mate, mating times and types in the swapped group related to Fig. 11.

contrast	estimate	SE	df	z.ratio	p.value
1 fix - 2 fix	0.207	0.062	Inf	3.339	0.01100
1 fix - 3 fix	0.347	0.059	Inf	5.895	<0.001
1 fix - 1 change	-0.212	0.460	Inf	-0.460	0.99700
1 fix - 2 change	0.068	0.460	Inf	0.149	1.00000
1 fix - 3 change	0.084	0.460	Inf	0.183	1.00000
2 fix - 3 fix	0.140	0.059	Inf	2.359	0.17100
2 fix - 1 change	-0.419	0.460	Inf	-0.910	0.94400
2 fix - 2 change	-0.139	0.460	Inf	-0.302	1.00000
2 fix - 3 change	-0.123	0.460	Inf	-0.268	1.00000
3 fix - 1 change	-0.559	0.459	Inf	-1.217	0.82900
3 fix - 2 change	-0.279	0.459	Inf	-0.608	0.99100
3 fix - 3 change	-0.263	0.459	Inf	-0.573	0.99300
1 change - 2 change	0.280	0.359	Inf	0.779	0.97100
1 change - 3 change	0.296	0.360	Inf	0.822	0.96400
2 change - 3 change	0.016	0.359	Inf	0.044	1.00000

Table 7 The result of the multiple comparison between latency to the first courtship, mating times and types in the swapped group related to Fig. 12A.

contrast	estimate	SE	df	z.ratio	p.value
1 fix - 2 fix	0.316	0.093	Inf	3.406	0.00865
1 fix - 3 fix	0.619	0.095	Inf	6.537	<0.001
1 fix - 1 change	-0.001	0.461	Inf	-0.002	1.00000
1 fix - 2 change	0.379	0.461	Inf	0.822	0.96354
1 fix - 3 change	0.324	0.451	Inf	0.717	0.97994
2 fix - 3 fix	0.303	0.094	Inf	3.209	0.01679
2 fix - 1 change	-0.317	0.463	Inf	-0.685	0.98364
2 fix - 2 change	0.063	0.463	Inf	0.135	0.99999
2 fix - 3 change	0.007	0.453	Inf	0.016	1.00000
3 fix - 1 change	-0.620	0.463	Inf	-1.339	0.76305
3 fix - 2 change	-0.240	0.463	Inf	-0.519	0.99544
3 fix - 3 change	-0.296	0.453	Inf	-0.652	0.98689
1 change - 2 change	0.380	0.402	Inf	0.944	0.93496
1 change - 3 change	0.324	0.391	Inf	0.829	0.96216
2 change - 3 change	-0.055	0.391	Inf	-0.142	0.99999

Table 8 Results of the GLMM model selection of behavioral transition probability in the mating test using fixed groups. ΔAIC (AIC full model – AIC null model) and Pr(>Chisq) were evaluated to reveal the effect of the mating experience and mating times between naïve and experienced males.

	AIC (full)	AIC (null)	deviance	deviance (null)	Chisq	Df	Pr(>Chisq)
Courtship -> courtship	515.29	510.24	495.29	504.24	8.95	7	0.26
Courtship -> wrapping	358.00	347.22	338.00	341.22	3.22	7	0.86
Wrapping -> wrapping rejection	246.97	239.82	226.97	233.82	6.85	7	0.44
Wrapping rejection -> courtship	222.78	215.31	202.78	209.31	6.54	7	0.48
Wrapping rejection -> wrapping	97.72	94.69	77.72	88.69	10.97	7	0.14

Table 9 Results of the GLMM model selection of behavioral transition probability in the mating test using swapped groups. ΔAIC (AIC full model – AIC null model) and Pr(>Chisq) were evaluated to reveal the effect of the mating experience and mating times between fixed and swapped conditions.

	AIC (full)	AIC (null)	deviance	deviance (null)	Chisq	Df	Pr(>Chisq)
Courtship -> courtship	101.38	98.67	87.38	94.67	7.30	5	0.20
Courtship -> wrapping	119.78	110.35	105.78	106.35	0.57	5	0.99
Wrapping -> wrapping rejection	78.11	70.42	64.11	66.42	2.31	5	0.81
Wrapping rejection -> courtship	72.37	65.24	58.37	61.24	2.87	5	0.72
Wrapping rejection -> wrapping	92.20	86.48	78.20	82.48	4.27	5	0.51

Table 10 Sample information of the RNA-seq

	Sample ID	Reads	Filtered reads	Alignment rate	RIN number
Naive 1	DRR346817	50737740	50737740	94.19%	10.00
Naive 2	DRR346818	48960510	48960510	94.06%	9.90
Post-naive 1	DRR346819	54432848	54432848	94.02%	9.90
Post-naive2	DRR346820	55046154	55046154	94.00%	10.00

Table 11 RNA-seq results. List of differentially expressed genes (DEGs) that were more highly expressed in post-naïve samples (2 matings) than in naïve samples. Genes are ordered from high to low expression using FPKM values as an index.

gene_id	gene_symbol	Log2|Fold Change|	FDR	FPKM (Naive)	FPKM (Pos-tnaive)
ENSORLG00000029600	*fkbp5*	1.877	1.510E-03	16.367	60.512
ENSORLG00000029251	***tshba***	2.660	1.491E-31	1.930	12.755
ENSORLG00000010690	***klf9***	1.166	6.180E-03	5.687	12.732
ENSORLG00000010816	***dio2***	1.064	2.275E-02	5.248	10.858
ENSORLG00000012432	interferon-induced, double-stranded RNA-activated protein kinase	2.262	3.058E-03	1.350	6.104
ENSORLG00000007145	*isg15*	2.381	1.272E-03	1.093	5.585
ENSORLG00000008101	*mov10*	1.067	1.244E-03	1.797	3.819
ENSORLG00000025334	novel gene	1.313	7.134E-03	1.582	3.447
ENSORLG00000025277	novel gene	1.712	5.343E-04	1.040	3.004
ENSORLG00000015847	*usp18*	1.443	5.558E-03	0.846	2.360
ENSORLG00000022172	*rnf213b*	2.115	6.958E-09	0.434	1.818
ENSORLG00000028533	novel gene	1.663	1.280E-02	0.719	1.809
ENSORLG00000026065	novel gene	1.340	2.745E-02	0.680	1.803
ENSORLG00000024839	uncharacterized protein PF11_0207-like	2.087	1.572E-04	0.362	1.573
ENSORLG00000015481	*mxb*	2.334	7.134E-03	0.279	1.471
ENSORLG00000014869	*cyp2n13*	1.147	7.963E-03	0.611	1.099
ENSORLG00000010672	*helz2*	1.753	6.166E-05	0.288	1.000
ENSORLG00000013082	*nlrc5*	1.630	4.943E-02	0.244	0.793
ENSORLG00000027804	interferon-induced protein 44-like	1.904	1.280E-02	0.172	0.718
ENSORLG00000028682	G2/M phase-specific E3 ubiquitin-protein ligase-like	1.479	2.767E-03	0.189	0.645

Table 12 RNA-seq results. List of differentially expressed genes (DEGs) that were more highly expressed in naïve samples than in post-naïve samples (2 matings). Genes are ordered from high to low expression using FPKM values as an index.

gene_id	gene_symbol	Log2|Fold Change|	FDR	FPKM (Naive)	FPKM (Pos-tnaive)
ENSORLG00000013094	*jarid2b*	-1.285	2.463E-43	288.672	119.023
ENSORLG00000005068	*dot1l*	-1.153	1.096E-05	123.739	55.427
ENSORLG00000030260	*rybpb*	-1.023	1.339E-14	37.428	18.389
ENSORLG00000013460	*smtla*	-3.309	8.735E-11	116.736	11.786
ENSORLG00000025409	novel gene	-1.005	7.715E-03	3.510	1.729
ENSORLG00000016221	*tlr5*	-1.583	3.612E-04	2.325	0.746
ENSORLG00000001455	*iqcb1*	-1.507	1.969E-05	1.836	0.710
ENSORLG00000012264	*vit*	-1.183	4.638E-02	1.556	0.694
ENSORLG00000025924	novel gene	-1.681	3.058E-03	0.758	0.139

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