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Analyse des rôles de différents domaines de la protéine Auts2a dans le contrôle maternel du comportement et caractérisation de l'impact d'un stress thermique sur la régulation de gènes de neurodéveloppement chez le medaka (Oryzias latipes)

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Table of contents

1. Context 1
1.1 AUTS2: the autism susceptibility candidate 2 gene 1
1.2 A large gene with multiple functions 2
1.3 Medaka development, from egg to larvae 4
1.4 Zebrafish and medaka behavior in cognitive analysis 5
1.5 AUTS2 project in the Fish Physiology and Genomics Laboratory (FPGL) 6
1.6 Aims of the internship 8
2. Materials and methods 8
2.1 Medaka and development conditions 8
2.2 Thermal stress experiment 10
2.3 Post larval phenotyping 12
2.4 Social behavior experiment 13
2.5 Statistical analysis 16
3. Results 17
3.1 Thermal stress experiment results 17
3.2 Post-larval phenotyping results 18
3.3 Social behavior experiment results 20
4. Discussion 21

4.1 Thermal stress experiment 21
4.2 Post larval phenotyping 23
4.3 Social behavior experiment 27
Conclusion 28
Bibliography 29

Glossary

aa: amino acids

ARV1: Fatty acid homeostasis modulator

ASD: autism spectrum disorder

AUTS2: activator of transcription and development regulator **bp:** base pairs

CM+: heterozygous mutant with maternal contribution of the *auts2* gene

CM-: heterozygous mutant without the maternal contribution of the *auts2* gene

CNS: central nervous system

CRMP: collapsing response mediator protein

CT: cycle threshold

C-terminus: carboxyl-terminus

DNA: deoxyribonucleic acid

dpf: day post fertilization **dph:**

day post hatching

DPYSL5: Dihydropyrimidinase like 5

ELZ: extreme left zone

ERZ: extreme right zone

FL-AUTS2: full-length AUTS2 protein

FPGL: fish physiology and genomic laboratory
HGNC: HUGO Gene Nomenclature Committee

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hpf: hour post fertilization

kb: kilo base **kbp:** kilo

base pair **KO**: knockout

mRNA: messenger RNA

NLS: nuclear localization signal

N-terminus: amino-terminus

OFT: open field test

PCR: polymerase chain replication

PLP2: proteolipid protein 2

PRR: proline rich region

RNA: ribonucleic acid

Rpm: round per minute

RT: room temperature

RT-qPCR: real-time quantitative polymerase chain replication

SD: standard deviation

SRR: serine rich region

WT: wild-type

Figures list

Figure 1: Examples of *AUTS2* syndrome's features in research animal models……...…1

Figure 2: Schematic representation of the *AUTS2* mRNA and its main described isoform.....2

Figure 3: Graphic representation of the early development stage of the medaka's egg…….…….4

Figure 4: Created lines for the *auts2* project at INRAE, FPGL……..…...…...….6

Figure 5: Graphic representation of a male medaka……...……...8

Figure 6: Schematic representationof the thermal stress experiment conducted in medaka……..….9

Figure 7: Schematic representation of the post-larval phenotyping method…….11

Figure 8: Social behavior setup for medaka……..……...13

Figure 9: Representation of the tracking area created with the EthoVision XT software in the middle aquarium of the experimentation device..……...15

Figure 10: Comparative expressions of *auts2a, arv1, dpysl5* and *plp2* in ovary and egg from thermal stressed medakas……...….16

Figure 11: Response of *auts2a* KO medaka's offspring post-larvae to light stimuli……..…17

Figure 12: Response of *auts2a* KO medaka's offspring to social stimuli……...19

Table list

Table 1: Example of experimentation plan for social behavior experimentation……..14

1. Context

1.1 *AUTS2:* the autism susceptibility candidate 2 gene

AUTS2 features

The *AUTS2*[1] gene is known as a regulator of neuronal development and involved in a wide range of psychiatric disorders such as schizophrenia, epilepsy, and autism spectrum disorder (ASD) (Hori & Hoshino, 2017). It has been identified in 2002, in a monozygotic pair of autistic twins with the similar t(7;20) (q11.2 ;p11.2) translocation and then firstly named as "autism susceptibility candidate 2" to recently become "activator of transcription and developmental regulator" according to the HUGO Gene Nomenclature Committee (HGNC) (Sultana et al., 2002). Patients carrying heterozygous *AUTS2* mutations display a variable phenotype known as the "*AUTS2* syndrome", which can be characterized by a severity score created in 2013 and based on 32 clinical features. The most frequently observed features in this score are feeding difficulties, intellectual disability, development delay, generalized hypotonia, strabismus, short and/or upturned philtrum, narrow mouth, scoliosis and tight heel cords (Beunders et al. 2013).

Auts2 in animal research models

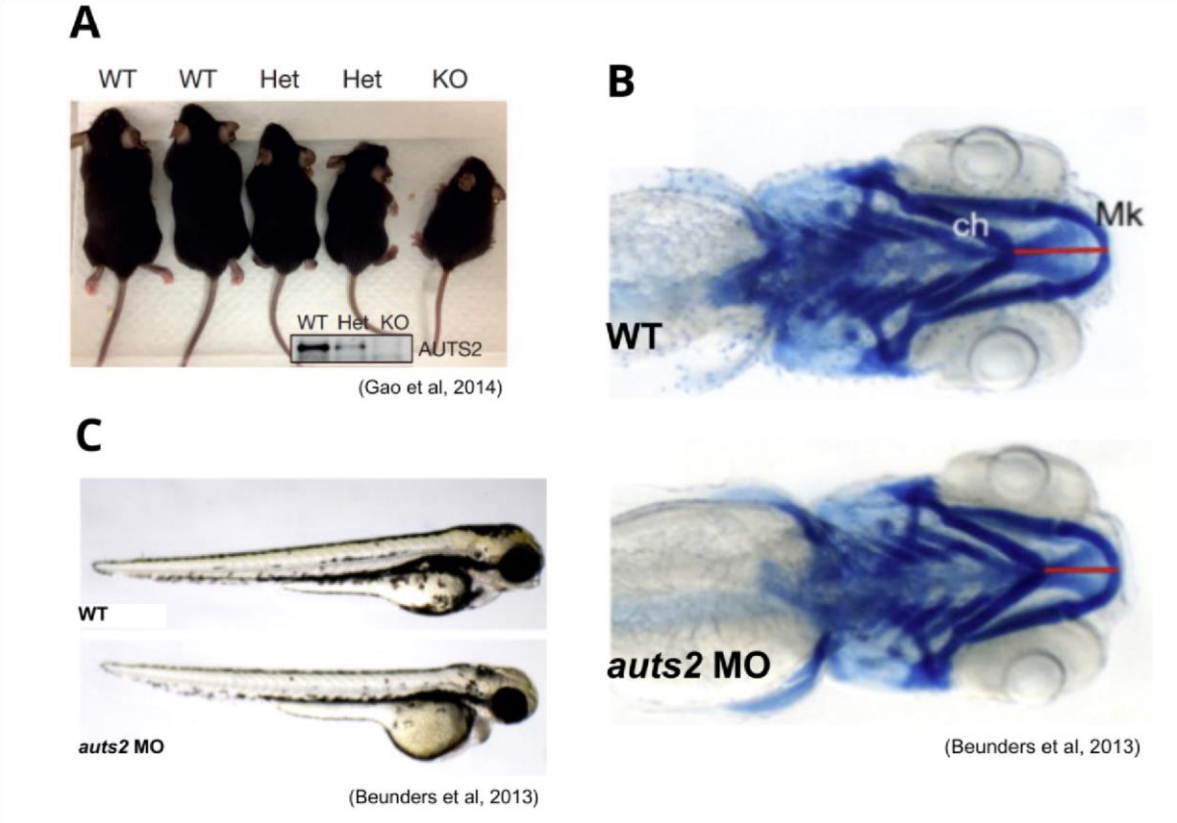

Figure 1: Examples of AUTS2 syndrome's features in research animal models.

[1] "*AUTS2*" gene symbol will be used for humans, "*Auts2*" for other mammals and "*auts2*" for fish.

A. *Body size comparison between wild type (WT), heterozygous mutant (Het) and knockout (KO) mice for auts2 (Gao et al, 2014).* ***B.C*** *Body and head size comparison between a zebrafish wild-type (WT) and a zebrafish injected with auts2 morpholinos (auts2 MO), (ch: ceratohyal, Mk: Meckel's cartilages) (Beunders et al, 2013).*

The link between *Auts2* mutation and neurodevelopmental disorders associated with specific features has also been shown in different animal research models. Heterozygous *Auts2* knockout (KO) mice display a body weight development delay (A, Figure 1) associated with righting reflex troubles and significantly less ultrasonic vocalization than wild types (WT) (Homozygous *Auts2* KO mice show the same phenotype in a stiffer form) (Gao et al., 2014). Depending on the mutation of the *Auts2* locus, heterozygous KO mice can also exhibit social deficit, altered anxiety and higher sensibility to auditory stimuli (Hori et al.,2020). A part of the AUTS2 syndrome features have also been described in zebrafish with *auts2* disruptions, such as smaller heads, eyes, bodies, pectoral fins than WT and craniofacial defects (B.C, Figure 1) (Nir Oksenberg et al., 2013. Beunders et al., 2013). However, despite the common use of teleost fish for biomedical analysis, there is a lack of *auts2* data in fish models.

1.2 A large gene with multiple functions

AUTS2 structure

The human *AUTS2* gene is composed of 1,195,032 base pairs (bp) made up of 19 exons. The first seven exons are separated by large introns (16 to 301 kilo base (kb)) while exons 7 to 19 are more compact (introns of 1 to 3 kb). The *AUTS2* gene encodes for a 1259 amino acids (aa) protein (FL-AUTS2, Figure 2) but many AUTS2[2] isoforms have been identified and especially a 711 aa protein (var1-AUTS2, Figure 2) and a 786 aa protein (var2-AUTS2, Figure 2) generated from alternative transcription sites and corresponding to the carboxyl-terminus part of the gene (C-terminus) (Beunders et al., 2013. Biel et al,, 2022). We find a similar exon structure of the AUTS2 gene within the vertebrates with different beginnings for the coding sequence, like in medaka and zebrafish where *auts2* starts after the exon 1 of the human *AUTS2* (Kondrychyn et al, 2017).

[2] "AUTS2" protein symbol will be used for humans and for every mammals and "Auts2" for fish.

AUTS2 identified functions

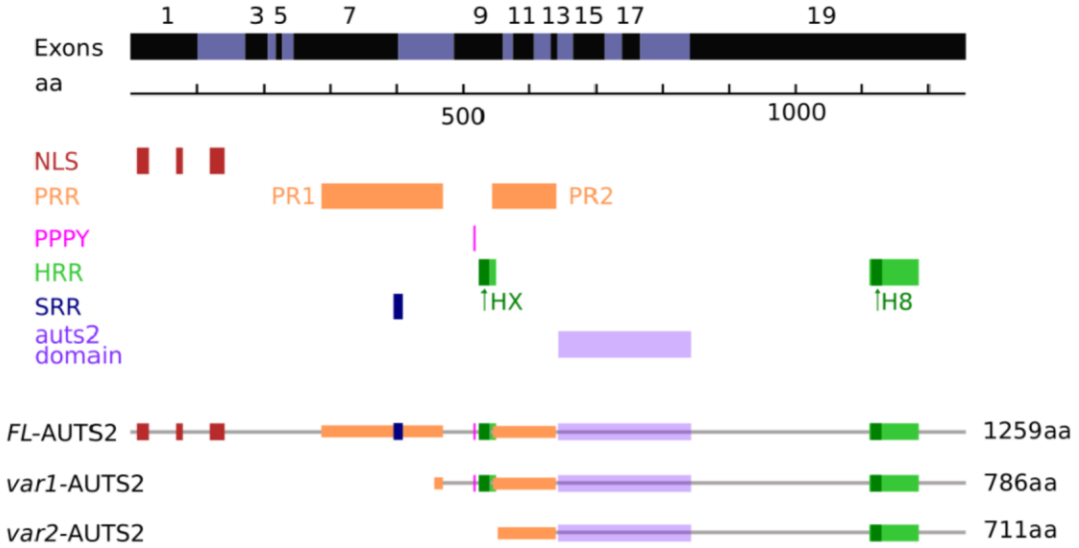

Figure 2: Schematic representation of the AUTS2 mRNA and its main described isoform, *(Clément, 2023). NLS: nuclear localization signal, PRR: proline rich region, PPPY: PY motif, HRR: histidine rich region, SRR: Serine rich region, FL-AUTS2: full length protein, var1-AUTS2 and var2-AUTS2: short isoforms of the AUTS2 protein.*

The AUTS2 structure is composed of several different motifs associated with identified functions. The amino-terminus (N-terminus) part of the protein includes three nuclear localization signals (NLS), a proline rich region (PRR) and a serine rich region (SRR) (Figure 2). In the other part of the protein we also find a PRR coupled with two histidine motifs and an “auts2 domain” which is characteristic of the Auts2 family (Beunders et al., 2013. Sultana et al., 2002). The AUTS2 protein displays features of a messenger ribonucleic acid (mRNA)-binding protein with stretch of repetitive aa motif and also highlighted in several studies where AUTS2 had been found binding protein complex (Hentze et al., 2018. Castanza et al., 2021). Several analyses have predicted that the AUTS2 proteins are likely to be localized in cell nuclei and especially for the full-length AUTS2 protein (FL-AUTS2) that displays NLS domains (Biel et al, 2022). In the nuclei AUTS2 acts as a transcription factor through the PRC1 complex that maintains the transcriptional repression of genes of the central nervous system (CNS) (Gao et al., 2012). Moreover, in the cytoplasm, it has been shown that the FL-AUTS2 is involved in the setting up of the cytoskeleton and especially in the axons’ elongation (Hori et al., 2014).

Auts2 identified functions in mammals and fish

All the features previously described on the *AUTS2* gene are also displayed by mice, zebrafish and medaka *Auts2* which correspond to a highly conserved structure within the vertebrates and witness of the main role of the gene in neurodevelopment. However, in teleost fish, we find two genes, *auts2a* and *auts2b*, resulting from the teleost specific whole genome duplication. It is known that *auts2a* is the most frequently retained gene and some species such as medaka display only the auts2a gene contrary to other species like zebrafish displaying both (Merdrignac et al, 2022).

AUTS2 expression

In humans, *AUTS2* is highly expressed in the fetal brain and also identified in skeletal muscle, kidney and in other organs with, however, a lower expression level (Oksenberg et al, 2013).

In medaka, *auts2a* expression has been highlighted in the developing brain and especially in the forebrain, midbrain, hindbrain and eyes. Moreover, maternally inherited *auts2a* had been detected in medaka's fertilized eggs before the beginning of the zygotic genome expression and before a radical increase of *auts2a* expression in the late embryonic brain formation (Merdrignac et al, 2022).

1.3 Medaka development, from egg to larvae

Reproductive biology of medaka

After an approximate growth period of nearly 4 months, medaka fish reach the adult stage marked by sexual maturation. Among female medaka, a daily production of eggs starts when they are kept under a reproductive photoperiod consisting of 16 hours of light followed by 8 hours in darkness. In fully mature medaka, oocyte maturation occurs every night and the ovulation appears just an hour before the onset of the light period. The female medaka holds oocytes in oviposition for a variable duration during which the male medaka fertilizes the egg. In their natural habitat, the eggs naturally detach from the female to undergo embryonic development. However, when medaka fish are maintained in aquariums, the fertilized eggs can be collected from the female and placed in petri dishes containing a specific medium. This method allows close monitoring of embryonic development. Moreover, the transparency of the eggs enables a detailed description of the embryonic development of medaka with its various development stages (Iwamatsu, 2004).

Early development stage of medaka

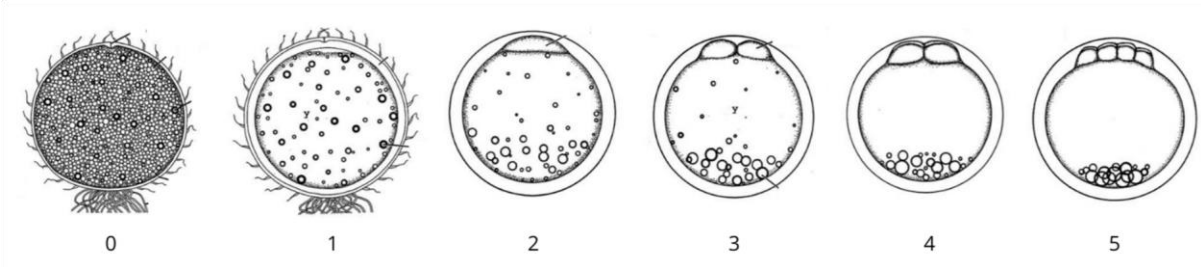

Figure 3: Graphic representation of the early development stage of the medaka's egg. *(Iwamatsu, 2004)**. Stage 0: unfertilized eggs. Stage 1: activated egg stage. Stage 2: blastodisc stage. Stage 3: 2 cell stage. Stage 4: 4 cell stage. Stage 5: 8 cell stage.*

Following the formation of the perivitelline space, which is initiated by the activation of the egg through the spermatozoon, pronuclei from the male and the female gather to form a zygote. Approximately 1 hour after fertilization at 26°C, the initial phase of the development starts with the onset of the first cell division (Figure 3).

As the early developmental processes unfold, organs are progressively formed to reach the hatching stage around ten days later, all under a consistent temperature of 26°C and in an adequate medium. The beginning of feeding behavior in medaka is not well-documented but it is established that the opening of the oral cavity to the mouth appears before the hatchin stage, approximately 6 days after fertilization (Iwamatsu, 2004).

Maternal factors in eggs

The early fish model development, as every animal development, is supported by proteins and maternal ribonucleic acids (RNAs) loaded into the oocyte during oogenesis. Before the zygotic genome activation, these maternal factors trigger fundamental processes within the egg which can be impaired by a variation of quality or quantity of mRNA and proteins. In recent years, there has been a growing focus on investigating the intergenerational impact of these maternal factors and especially a focus on molecular mechanisms and gene expression.

Various types of maternal factors exist, including RNA, proteins, hormones…and these represent a wide fraction of the coding part of the genome. For example, in zebrafish, maternal factors within the egg account for 75% of the entire genome (Vastenhouw et al., 2019). Moreover, in zebrafish, almost 25% of the zygotic genome is expressed during the zygotic activation genome period and these activated gene codes for transcription factors or development regulators fundamental for the maternal mRNA degradation (Aanes et al., 2011. Lee et al., 2013. Harvey et al., 2013).

The repartition of the maternal factors in the egg has also a key role in the setting up of the developing plan of the embryo through a variable cytoplasmic composition and then a precise regulation of gene expression.

1.4 Zebrafish and medaka behavior in cognitive analysis

Behavior analysis are widely used in fish models to identify phenotypes linked to the genetic approach of the model. Several standardized tests have emerged recently and are currently used to explore different aspects of fish behavior such as anxiety behavior or social behavior.

Open field test (OFT)

OFT is one of the main used behavior tests. Responses of fish behavior to a novel environment are measured through the locomotory or exploratory activity and a reduction of these responses is defined as habituation. OFTs are used to analyze cognitive responses of fish like anxiety or learning abilities through the habituation to a repeated exposure to the new environment (Matsunaga, 2010). Thigmotaxis (avoiding the center of the area) is a behavior observed during these tests to assess the anxiety-like behavior of fish. Anxious subjects will swim closer to the edges of the area than other ones. This behavior had been noticed in model fish and especially in zebrafish and medaka.

Contrary to medaka larvae, which do not show a typical thigmotactic behavior, adult medaka spend much more time swimming close to the side of a new environment than in the center. However, adult medaka behaviors are fluctuant with a short period of high exploratory behavior at the beginning of an OFT followed by a thigmotactic behavior with a spatial preference for edges (Lucon-Xiccato, 2020). Likewise, larvae and adult zebrafish are likely to avoid the center of the tank when they are exposed to a new surrounding (Schnörr et al, 2011).

Light stimuli response

Another way to analyze the cognitive responses of fish is to observe the scototaxis (dark/light preference) or responses to light stimuli. Subjects are exposed to an empty tank with a white and a black area. The main part of model fish have shown a significant preference for the dark

part of the tank and a rise of the activity in the dark part is known as a witness of an anxiety-like behavior in opposition to an increase of the activity in the white part which reflects an antianxiety-like behavior (Maximinio, 2010).

Even if this scototaxis protocole had been validated to investigate the anxiety response of fish, it depends on the natural preference for the subject for black or white areas. And even if some studies have shown that zebrafish, goldfish and guppies are more likely to stay in the dark, for other species of model fish like medaka, discussions are still being held and especially because of different results at different development stages. Black areas are usually known as a safe place for fish where they can hide from potential predators, however, in opposition to adult zebrafish, the adult medaka (10-12 months) do not display an avoidance for white areas (Lucon-Xiccato, 2022).

Social behavior

Social behavior tests are also often used to characterize the cognitive response of model fish. In the most common test, subjects are tracked in an aquarium with, on one side, another aquarium with a group of conspecifics and on the other side, an empty aquarium. Sociability of the subject is assessed by measuring the time spent on the side with the social stimulus. As it has been highlighted in several studies, medaka shows an attraction to social stimuli with possible sophisticated social interactions through the recognition of group mates (Lucon-Xiccato, 2022. Wang, 2017).

These behavioral tests are widely used means to investigate behavioral phenotypes in fish models but it has been shown that the results are also extremely dependent on the experimental settings. For example, the risk-taking behavior of the mosquitofish strongly depends on the size of the arena and on the ontogenetic stage (Polverino, 2016).

1.5 AUTS2 project in the Fish Physiology and Genomics Laboratory (FPGL)

Thermal stress and cognitive responses impairment

In 2019, a research team led by Julien Bobe (INRAE, FPGL) initiated a study to investigate the intergenerational impact of maternal stress on cognitive response in the offspring. The aim was also to focus on the underlying mechanisms through investigating neurodevelopmental gene expression within eggs. They have shown that a maternal temperature exposure in rainbow trout triggers impaired emotional and cognitive responses in the offspring. Indeed, alevins from thermally stressed mothers displayed a decreased anxiety and weaker learning abilities than controls. Moreover, several neurodevelopmental genes have been highlighted as dysregulated within eggs from stressed fish. Among these dysregulated genes they found *arv1*, *plp2* or *dpysl5* and especially *auts2* (Colson et al.,2018). Even if we cannot directly associate the impairment of auts2 expression within eggs and the behavioral phenotype observed in offspring, we can remember that similar behavior disorders were displayed by other animal research models with *Aust2* mutation.

The auts2 project

This previous study emphasized the impact of maternal stress on egg quality and offspring behavior but understanding the underlying mechanisms remains limited and knowledges are

scarce. Then, following the initial study, the research team started to investigate these mechanisms focusing on auts2 and utilizing the medaka as a research model. They had created several lines of medaka with distinct mutations of *auts2* in order to characterize the impact of the maternal auts2a contribution in neurodevelopment and behavior of the offspring.

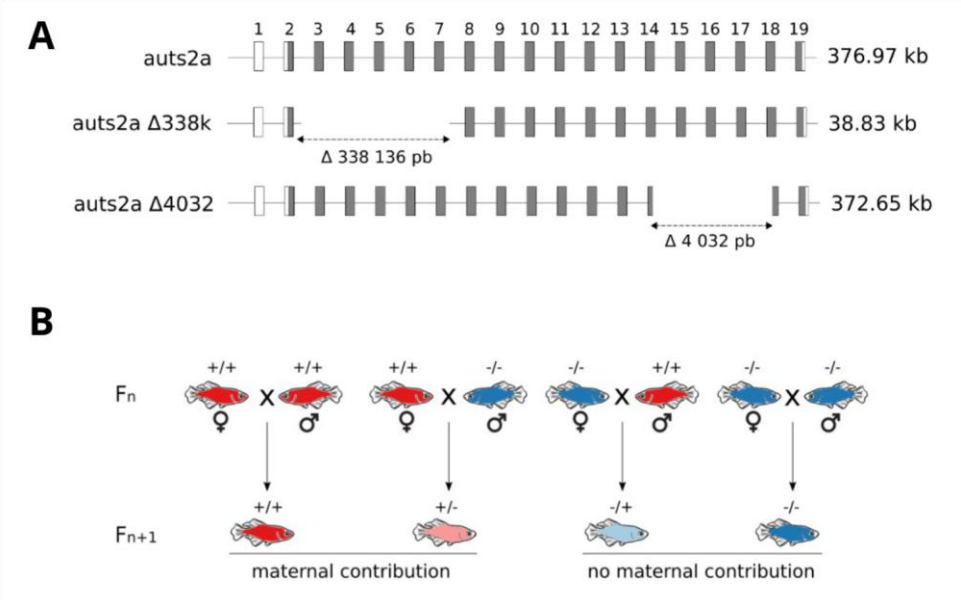

Figure 4: Created lines for the auts2 project at INRAE, FPGL, *(Clément, 2023).*
A: *auts2a deletions made to create two lines (Δ338K and Δ4032).* ***B:*** *crosses made to study the impact of maternal aust2 contribution.*

Two different lines have been created using CRISPR/Cas9, characterized by two distinct mutations. The first one, named Δ338K, corresponds to a deletion of almost 90% of the gene (338 kilo base pairs (kbp)) in the N-terminus part of the gene starting at the exon 2 to end at the exon 7. The second one, named Δ4032, corresponds to a deletion of 4032 bp in the C-terminus part of the gene, starting at the exon 14 to end at the exon 18 (A, Figure 4). For each line, homozygous and heterozygous medakas have been crossed to obtained in one hand, wild-type (+/+) and heterozygous (+/-) with maternal contribution of the gene (named “CM+”) and on the other hand, homozygous (-/-) and heterozygous (-/+) without maternal contribution of *auts2a* (named “CM-”) for both of the gene mutation (B, Figure 4).

Previous outcomes

In fish from the Δ338K line, it has been shown that the lack of maternal contribution of auts2a is responsible for a lower hatching rate, smaller heads and smaller bodies. Moreover, in the same line, adult fish without maternal contribution displayed a reduced anxiety-like behavior and a worst recognition of the environment, in comparison to fish with maternal *auts2a* contribution. Smaller heads and smaller bodies have also been highlighted in fish without the maternal contribution from the Δ4032 line, however, no behavioral phenotype was described in this line. Moreover, no behavioral test has yet been done on larvae from these lines and there is also still a lack of data on social behavior of these two lines as we know that social trouble are described in KO mice for auts2.

1.6 Aims of the internship

Within this context, the primary objective of my internship was to investigate the expression of neurodevelopmental genes in mother medaka subjected to thermal stress. The purpose was to determine whether the findings previously observed in trout hold true for medaka as well.

The second objective of this project was to characterize post-larval behavior phenotypes linked with *auts2a* mutations. The aim was, first, to conduct a comparative analysis of these phenotypes in order to determine the maternal effect of *auts2* on post-larval behavior for these two lines. Furthermore, a secondary purpose was to compare these phenotypes according to the localization of the deletion within the gene and try to clarify the functions of the distinct protein segments through existing published insights.

Finally, the last objective of this internship was to characterize the auts2a maternal contribution on social behavior in fairly mature medaka. In the same way as the previous purpose, this objective also encompassed a comparative analysis between the distinct lines to know whether the mutation's localization within the gene affects the behavior of the offspring.

To address these objectives, we conducted 3 separate experiments that we carried out all along the duration of this internship, a thermal stress experiment,a post larval-phenotyping and a social behavior experiment (in the same order as the objectives).

First of all, we will examine the materials and methods employed for each distinct experiment. Following this, we will explore the results acquired and, in another part, discuss these outcomes. Lastly, we will conclude by addressing the inquiries associated with the three primary objectives of this internship.

2. Materials and methods

2.1 Medaka and development conditions

Medaka as a fish research model

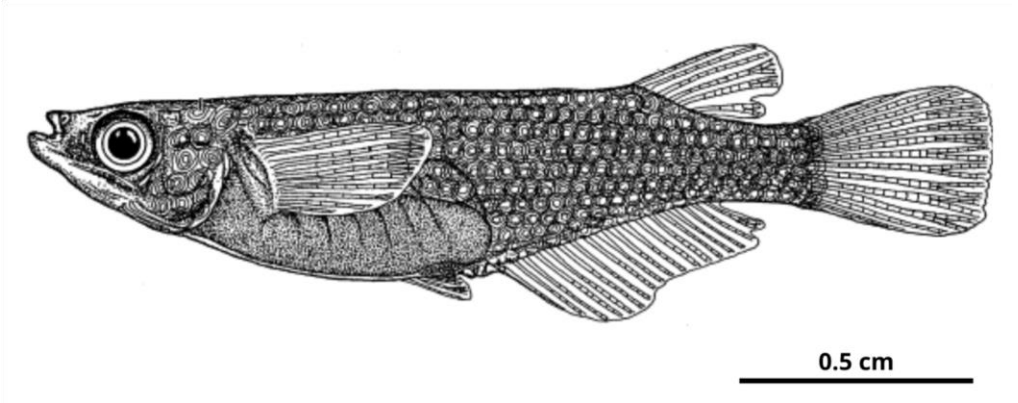

Figure 5: Graphic representation of a male medaka, *(Iwamatsu, 2004).*
This representation corresponds to the stage 44 of the normal development table in medaka created by Iwamatsu in 2004.

Medaka is a freshwater species from Asia associated with a widespread use in fishkeeping as its body size does not reach more than 5 cm at mature age. Moreover medaka displays a wide range of features which made it a fine subject for fish experimentation and as an animal model for genetic investigations.

After almost 3 months of development, females medaka start to spawn about thirty oocytes every morning for an external fertilization by males. The large number of eggs generated provides convenience to elaborate new lines or to get new subjects for analysis.

The spawning can be initiated by regulating the light period at an optimal temperature (26°C) from a growth photoperiod (8 hours light and 16 hours dark) to a reproduction photoperiod (16 hours light and 8 hours dark) (Koger et al., 1999). These translucent eggs can be collected on the female's genital papilla and kept at 26°C to follow the embryonic development.

Moreover, that species shows a sexual dimorphism with a longer anal fin in male and even if this dimorphism is not evident at early ontogenetic stages, it is also possible to identify the sex of the subject using the sex-determining gene (Matsuda et al, 2002).

In the context of the *auts2* project, medaka had been chosen as it is an easy to breed species used in many different studies and as it displays only the *auts2a* gene contrary to zebrafish. Sequence analysis have also shown that the *auts2a* gene in medaka corresponds to the *AUTS2* gene in humans in which *AUTS2* features have the most been described. It is also a species with a fully characterized genome recently updated.

Furthermore, an increasing number of research studies are being conducted using this model fish species, focusing particularly on its behavioral reaction following exposure to chemical products. Consequently, behavior of these fish has been partially characterized and standardized tests have been developed to explore their cognitive responses.

Medaka in INRAE fish facility

In this work every fish with more than 12 days post-fertilization (dpf) was kept in aquariums with a consistent water renewal at 26°C, and fed 3 times a day. In fish facility, medakas are exposed to a light about almost 300 lux and with 2 different photoperiods, 8 am to 11.45 pm for reproductive phase and 8 am to 8 pm for growth phase. Densities in petri dishes and in aquariums are variable. Eggs densities within petri dishes were maintained at high levels to facilitate hatching induction in the medium. After hatching, densities were adjusted according to the size of developing larvae (approximately 0.5 cm for 1 day post-hatching (dph) larvae and approximately 1 cm for 21 dph post-larvae).

2.2 Thermal stress experiment

Experimental setup and sample collection

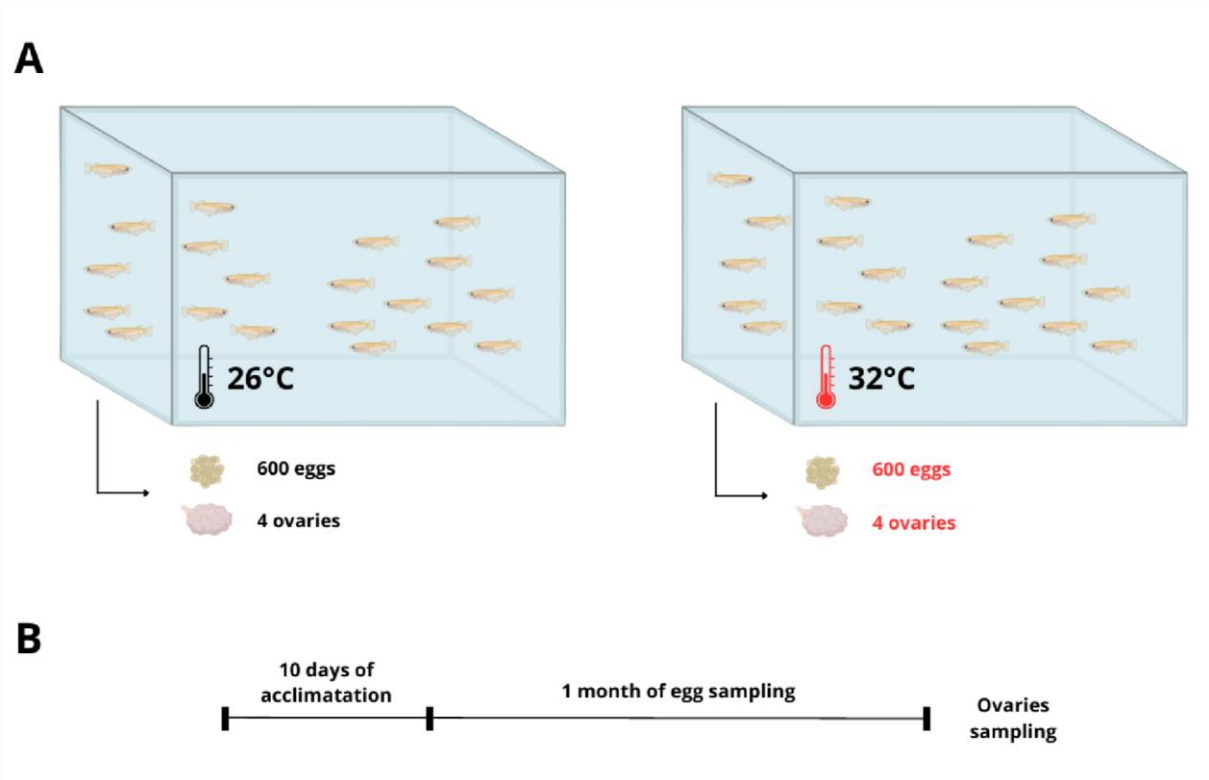

Figure 6: Schematic representation of the thermal stress experiment conducted in medaka.
A: *12 females and 8 males were placed in two different temperature conditions (26°C and 32°C). For each aquarium 600 eggs were collected and 4 ovaries. B: after 10 days of acclimatization to the new environment and to the thermal conditions, we started to sample eggs during 1 month. Ovaries were collected following the egg sampling.*

To investigate the effect of thermal stress in medaka on gene expression, 2 aquariums were set up in an isolated room with 2 different temperature conditions, one with a normal temperature, about 26°C, and another one about 32°C (which represents a temperature increase of 23%) (Figure 6, A). In each aquarium, 8 males and 12 females from the same previous development conditions were placed. As other fish in the fish facility, they were fed 3 times a day and bred with a consistent water renewal.

Sample collection

After 10 days of habituation to the new environment for each aquarium, we started to sample eggs during one month to reach an amount of 600 eggs per temperature condition (Figure 5, B). Eggs were collected on females every morning of week days and cleaned from attaching filament before being saved in cryotubes at -80°C. The aim was to collect non fertilized eggs or eggs with a maximum of 1 cell to investigate analysis on the maternal factor in the egg and to avoid zygotic gene expression (Figure 3).

After egg sampling, females were euthanized using a bath with specific concentration of tricaine methane sulfonate and bicarbonate. For each aquarium, 4 ovaries were collected and saved at -80°C in cryotubes as every sample.

RNA extraction

Eggs were pooled by 100 to get 6 replicates per temperature conditions and as ovaries, transferred still frozen into Precellys Lysing Kit - Soft Tissue Homogenizing CK14 2 mL tubes (Bertrin Technologies, France) with 1.5 mL of TRIzol (TRI Reagent, Molecular Research Center, Cincinnati). Samples were then homogenized in the Precellys homogenizer (Bertrin Technologies, France) with a 6*30 seconds at 6,800 rounds per minute (rpm) program. After 10 minutes of incubation at room temperature (RT), tubes were short spined and transferred into 2 mL RNase-free Eppendorf tubes.

After this first step, 300 µL of chloroform were added, tubes were shaken for 15 seconds and incubated at RT for 10 minutes. After half an hour of centrifugation at 12,000 g and at 4°C the RNA phase was extracted from tubes to new 1.5 mL RNase-free Eppendorf tubes. We added 500 µL of isopropanol for a 10 minutes incubation at RT followed by a centrifugation for 30 minutes at 12,000 g and at 4°C. Then the supernatant was removed and 1 mL of ethanol was added for a second centrifugation phase at the same previous settings (we repeated this step a second time). After that, supernatant was removed to let the cheek dry and almost 50 µL of nuclease-free water was added in each tube (depending on the size of the cheek) to dilute the RNA's cheek. We managed to extract RNA for every ovary samples (4 per temperature conditions), and for only 4 of the 6 egg samples.

Reverse transcription

RNA concentration was analyzed for each sample using the ND-1000 Spectrophotometer (NanoDrop Technologies, USA) to determine the volume to extract to get 1 µg of RNA into nuclease-free water for a final volume of 10 µL. Then, into RNase-free tubes on ice, we added 4 µL of 5X Reaction Mix (Thermo Fisher Scientific, USA), 2 µL of Maxima Enzyme Mix (Thermo Fisher Scientific, USA), 4 µL of nuclease-free water and 10 µL of the RNA solution of one sample. Then, these tubes were placed in the SimpliAmp Thermal Cycler (Thermo Fisher Scientific, USA) with the Maxima enzyme program to get a reverse transcription of our RNA samples for real-time quantitative polymerase chain replication (RT-qPCR).

In order to control the quality of our extraction we have done 2 more tubes with a sample of each temperature condition and with the exact same protocol but with nuclease-free water instead of enzyme. An amplification signal with the RT-qPCR of tubes will be indicative of a DNA contamination in our samples.

Real-time PCR

We targeted 4 genes described as dysregulated in eggs from thermal stresses rainbow trouts through RT-qPCR: *auts2a*, *dpysl5*, *arv*1, and *plp2.* To target these genes we used 20 mer primers elaborated using Primer3 (Rozen & Skaletsky, 2000).

All samples were analyzed in triplicates with, in each well of the qPCR plate, 4 µL of a 1/20 dilution of the reverse transcription solution, 5 µL of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA) and 1 µL of solution with reverse and forward primers at 5 µM (Controls were analyzed the same way but in duplicates). To normalize the signal we used two ribosomal DNA: 5S and 18S with the same protocol but with a 1/2000 dilution of the reverse transcription solution and only with a 2 µM primers solution. Plates were placed into the StepOnePlus RealTime PCR System (Thermo Fisher Scientific, USA) with a set program for this mix.

2.3 Post larval phenotyping

Experimental setup

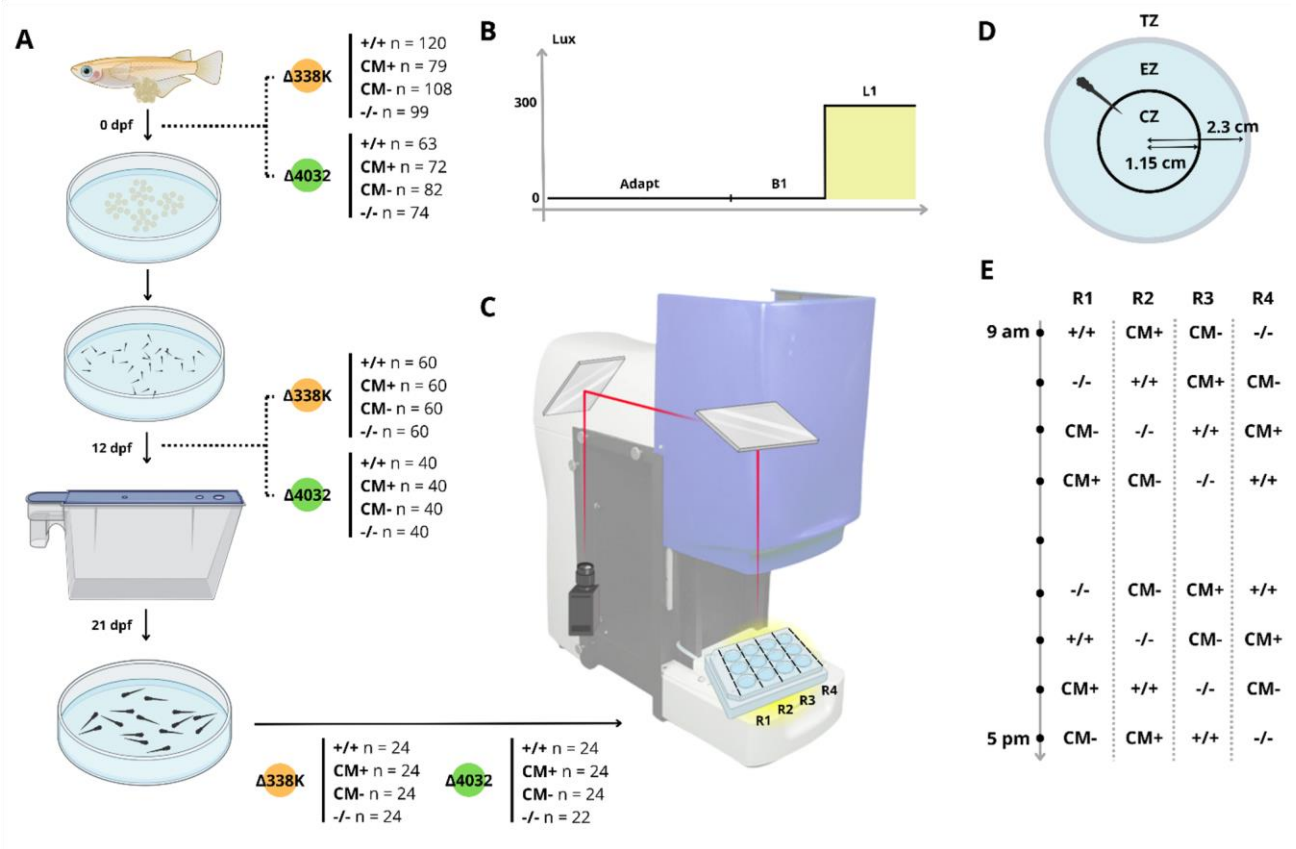

***Figure 7: Schematic representation of the post-larval phenotyping method**.*
***A:** Embryo raising for each genotype and for each line. **B:** light phases in the observation chamber of the Zebrabox (ViewPoint, France). C: Schematic représentation of the Zebrabox (ViewPoint, France. D: Zoom on a well of a plate with a post-larvae (CZ: Central Zone, EZ: External Zone, TZ: Total Zone). E: Plates plan of the experimentation day.*

Egg collecting and larval development

Eggs were collected from mothers kept in 5L aquariums with a ratio of 5 females for 3 males. After spawning on a friday, about 60 to 120 eggs were cleaned from attaching filament and gathered by genotype in petri dishes in an incubator at 26°C with natural photoperiod and in an embryo raising medium (water, 0.1% NaCl, 0.003% KCl, 0.004% CaCl2, 0.016% MgSO4 and few drops of methylene blue dye).

After 12 days in the incubator with a medium renewal every weekday, post-larvae were gathered in 1L aquarium per genotype with variable densities from 40 to 60 post-larvae/L in the fish facility of the lab and started to be fed (A, figure 7). Then, at 21 dpf, post-larvae were collected in petri dishes with water from the animal facilities and kept at 26°C during the whole day of behavior experimentations. The two lines investigated in this work (Δ338K and Δ4032) have been studied within 1 week interval but with the same protocol.

Experiment protocol

Every hour of the experimentation day, from 9 am to 5 pm, fish were picked randomly from petri dishes and loaded into 12 wells cell culture plates (Costar, USA) following a previously defined loading plan to avoid a maximum of experimentation bias (E, figure 7).

Plates were previously kept in the incubator at 26°C to cap temperature discrepancy and filled with water from fish facilities at the same temperature. Just after loading with water, plates were loaded with post-larvae and directly placed in the Zebrabox device (ViewPoint, France). Background detection was reset after the observation chamber closing at the beginning of each test and tracking threshold was kept at the same level for each post-larvae tracked.

Tracking software and Zebrabox protocol

In the tracking software, 12 wells of 2.3 cm were created according to the size of the plate used. For each plate, we designed artificial tracking zones and especially a central zone 2 times smaller than the total zone in order to get an external zone about the length of 21 dpf post-larvae (CZ, TZ and EZ on D, figure 4).

Each plate was tracked for 20 minutes preceded by an adaptation phase of 20 minutes in darkness according to results previously found in medaka (Chiffre et al., 2014). The tracking period is separated in 2 distinct phases linked to light stimuli and adapted from previous studies about locomotion and behavior essay (Chiffre et al., 2014. Le Bihanic et al., 2014). First 10 minutes were tracked in darkness and the 10 last minutes were tracked with a light exposure of about 300 lux (similar to the light power measured in the fish facility of the lab).

All plates were kept at 26°C during each test using a thermo-regulator module (ViewPoint, France) to avoid temperature effects. Moreover, the Zebrabox device was placed into an isolated room to ensure a quiet environment and to limit external bias.

Post-processing data

Multiple integration periods of 1 minute were set during the 20 minutes period of tracking. Paths of post-larvae for each minute were observed to guarantee the quality of tracking and to withdraw possible tracking interferences. We have summed the integration periods according to light phases in order to observe data usually investigated in locomotor and behavioral tests such as: total distance moved, time spent in the central zone and entries number in the central zone (Chiffre et al, 2014. Lucon-Xiccato, 2020).

2.4 Social behavior experimentation

Fish housing in the fish facility

Social behavior investigations were performed using 3-month-old medakas raised at equivalent densities and kept in 10 L aquariums in fish facilities of the lab (2 aquariums per genotype and per line with 23±1 fish). They were kept exposed to a growth photoperiod at 26°C with water renewal and fed three times a day before experimentation. In experimentation's week, fish were fed in the morning and in the evening after the last trial of the day. The 2 lines used in that work have been investigated with 1 week interval but with the same protocol and the same experimentation plan.

Experimental setup

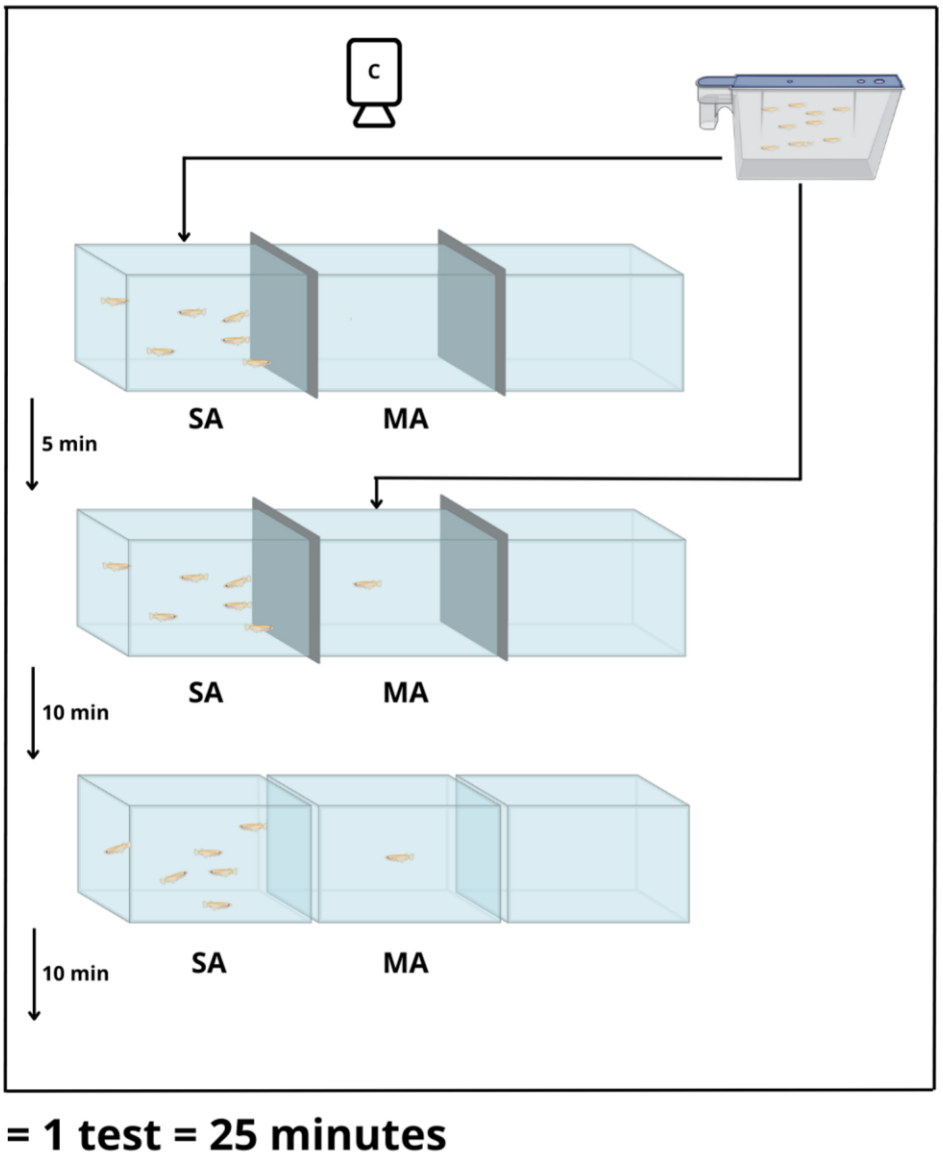

Figure 8: Social behavior setup for medaka.
Fish are extracted from housing aquariums to be placed in the experimental setup made of 3 distinct aquariums isolated by 2 opaque separations. The middle aquarium (MA) is the only recorded area in this experiment and is the tracked area. The shoal aquarium (SA) acts as the social stimuli for the subject.

Social behavior tests of a line were done over 2 weeks with 4 days of experimentation per week and with 6 tests per day (1 genotype per test) (Table 1). In a test, 6 fish were picked randomly from the keeping aquarium and placed in the shoal aquarium of the experimental installation for an adaptation phase of 5 minutes (Figure 8). Then, another fish was picked randomly in the aquarium to be placed in the middle aquarium (according to the sex needed to get the same number of male and female tested per genotype). After 10 minutes of adaptation, dividing plates between windows of the aquariums were removed for a 10 minutes test.

At the end of a test, water of each experimental aquarium with fish was changed and another genotype was used for another test according to the experimentation plan. Trials were run

between 2 pm to 5 pm in an isolated room at 26°C and after each experimentation, fish were euthanized for a fin clipping in order to assess sex ratio and confirm genotype.

Experimentation plan

***Table 1: Example of experimentation plan for social behavior experimentation**.*

Serie	Line	Date	Hour	Genotype
1	4032	10-juil	14h - 14h25	+/+
2	4032	10-juil	14h30 - 14h55	CM+
3	4032	10-juil	15h00 - 15h25	CM-
4	4032	10-juil	15h30h - 15h55	-/-
5	4032	10-juil	16h00 - 16h25	+/+
6	4032	10-juil	16h30 - 17h00	CM+
7	4032	11-juil	13h38 - 14h03	CM-
8	4032	11-juil	14h08 - 14h33	-/-
9	4032	11-juil	14h39 - 15h04	+/+
10	4032	11-juil	15h09 - 15h34	CM+
11	4032	11-juil	15h39 - 16h04	CM-
12	4032	11-juil	16h08 - 16h34	-/-
13	4032	12-juil	14h - 14h25	-/-
14	4032	12-juil	14h30 - 14h55	CM-
15	4032	12-juil	15h00 - 15h25	CM+
16	4032	12-juil	15h30h - 15h55	+/+
17	4032	12-juil	16h00 - 16h25	-/-
18	4032	12-juil	16h30 - 17h00	CM-
19	4032	13-juil	14h07 - 14h32	CM+
20	4032	13-juil	14h37 - 15h02	+/+
21	4032	13-juil	15h08 - 15h33	-/-
22	4032	13-juil	15h38 - 16h03	CM+
23	4032	13-juil	16h08 - 16h33	CM-
24	4032	13-juil	16h38 - 17h04	+/+

Serie	Line	Date	Hour	Genotype
25	4032	17-juil	14h - 14h25	CM-
26	4032	17-juil	14h30 - 14h55	-/-
27	4032	17-juil	15h00 - 15h25	+/+
28	4032	17-juil	15h30h - 15h55	CM+
29	4032	17-juil	16h00 - 16h25	CM-
30	4032	17-juil	16h30 - 17h00	-/-
31	4032	18-juil	14h10 - 14h35	+/+
32	4032	18-juil	14h40 - 15h05	CM+
33	4032	18-juil	15h12 - 15h37	CM-
34	4032	18-juil	15h42 - 16h08	-/-
35	4032	18-juil	16h12 - 16h37	+/+
36	4032	18-juil	16h43 - 17h08	CM+
37	4032	19-juil	14h - 14h25	CM+
38	4032	19-juil	14h30 - 14h55	+/+
39	4032	19-juil	15h00 - 15h25	-/-
40	4032	19-juil	15h30h - 15h55	CM-
41	4032	19-juil	16h00 - 16h25	CM+
42	4032	19-juil	16h30 - 17h00	+/+
43	4032	20-juil	14h17 - 14h43	-/-
44	4032	20-juil	14h48 - 15h13	CM-
45	4032	20-juil	15h19 - 15h45	CM+
46	4032	20-juil	15h51 - 16h16	+/+
47	4032	20-juil	16h21 - 16h46	-/-
48	4032	20-juil	16h52 - 17h17	CM-

In order to avoid potential experimental bias, we devised a specific experimentation plan in which each genotype is subjected to testing every hour of the experimentation day, ensuring equal frequency as other genotypes. The 2 investigated lines in this work have followed the exact same plan spread on 8 days (Monday, Tuesday, Wednesday and Thursday of 2 weeks for the Δ4032 and Monday, Tuesday, Wednesday and Friday of 2 weeks for the Δ338K).

Record device and analysis

Experiments trials were captured using a camera device positioned above the experimental aquarium. For each trail, a fresh video recording was initiated. These video recordings were gathered at the conclusion of an experimental week and subsequently transferred to a computer equipped with video editing software. Videos were then cropped and converted into a compatible file format supported by the analyzer software. Analyses were conducted using the EthoVision XT software (Noldus, Netherlands).

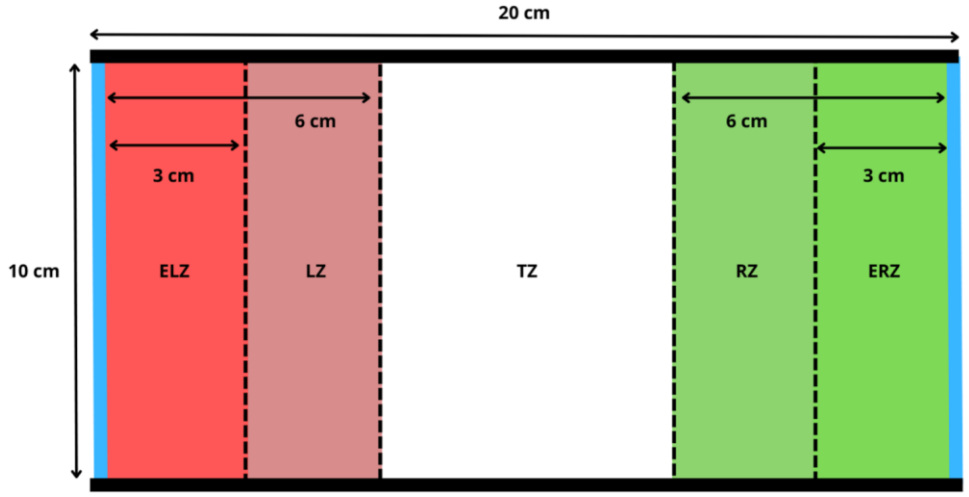

Figure 9: Representation of the tracking area created with the EthoVision XT software in the middle aquarium of the experimentation device.
ELZ: Extreme Left Zone, LZ: Left Zone, TZ: Total Zone, RZ: Right Zone, ERZ: Extreme Right Zone. Blue lines symbolize glasses in the middle aquarium of the experimentation device.

For each video, 5 tracking zones were created: 2 on each side of the aquarium and one for the total zone of the aquarium incorporating each zone (TZ in Figure 9). The extreme zones are included in the bigger zone and have been set to correspond to a contact between the subject and the window contrary to the larger one (ELZ, ERZ, LZ and RZ in Figure 9). These zones have also been set according to the size of observed fish in order to have an extreme zone much bigger than the fish and to avoid tracking bias. The area settings were calibrated according to the settings of each video recording, however size and proportion of the tracking area were kept unchanged for each test. The tracking thresholds were set individually for each video to avoid interferences and to fit with the video settings. The beginning of the tracking period was defined as the instance when the fish initially entered the ELZ. This decision was based on our observation that, upon the removal of separations, some fish tended to remain within the RZ and potentially overlooking the social stimuli present in the other part of the aquarium. Consequently, we decided to analyze the behavior of fish during 5 minutes after the beginning of the tracking.

2.5 Statistical analysis

Statistical analyses were performed using RStudio Statistical software version 1.4.1106 (The R Foundation for Statistical Computing, Austria).

For thermic stressed experiments, each gene was investigated separately. We used a Student test on data following a normal distribution to investigate the effect of temperature in gene expressions. For other data, we used a Wilcoxon test.

In post-larval phenotyping's experiments, for each observed variable, light phases were treated separately and genotypes were pairwise compared. As the large majority of data were not normally distributed, we used a Wilcoxon test to investigate significant differences between genotype within phases.

For the social behavior analyses, for each variable, genotypes were pairwise compared using a Wilcoxon test as data do not display a normal distribution.

3. Results

3.1 Thermal stress experiment results

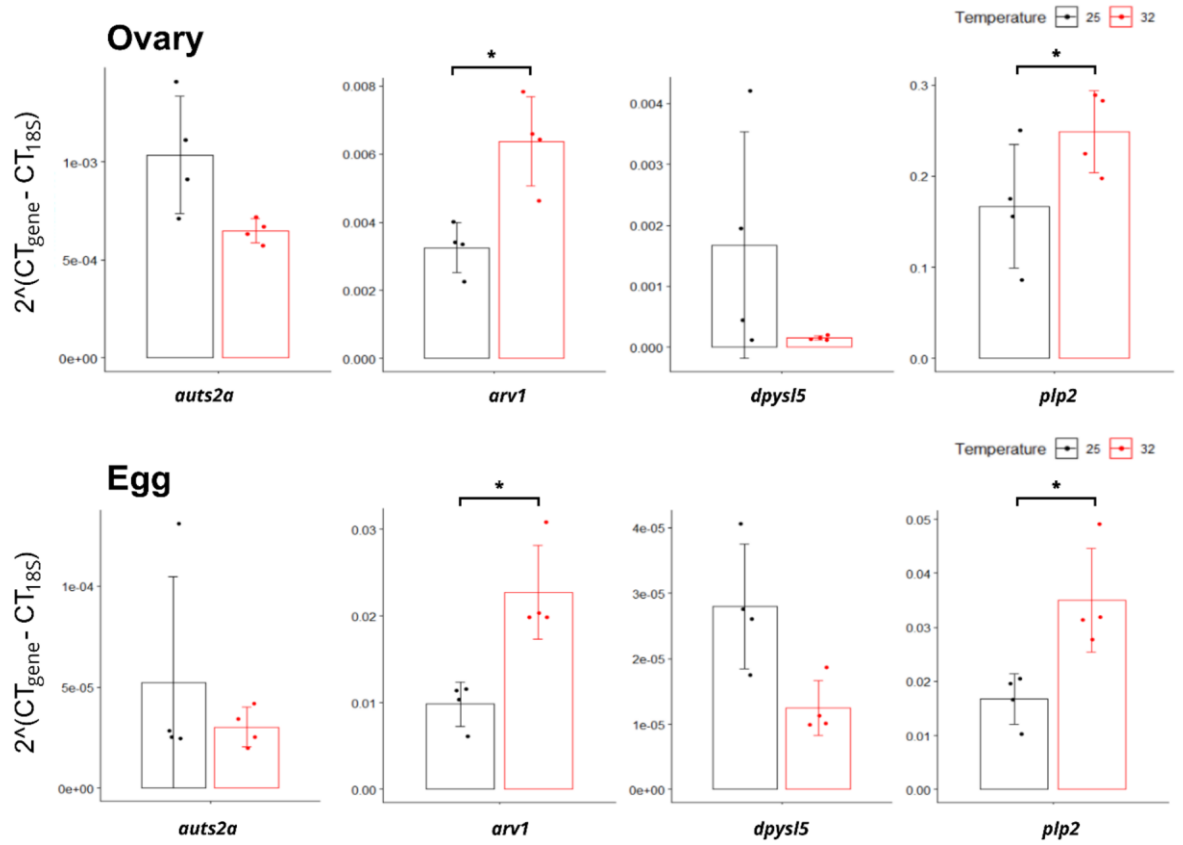

Figure 10: Comparative expressions of auts2a, arv1, dpysl5 and plp2 in ovary and egg from thermal stressed medakas.
*The expressions of 4 genes have been normalized using a ribosomal gene (18S) and fold expressions are investigated using ΔCycle Threshold (CT) comparisons ($\Delta CT=CT_{gene}-CT_{18S}$).Values are reported as mean ± standard deviation (SD) and *P < 0.05 indicates significant differences between gene expression.*

arv1 and plp2

In contrast to *auts2a* and *dpysl5*, we observed a significant differential expression in *arv1* and *plp2* within both ovaries and eggs from mothers exposed to thermal stress. Both are up-regulated in the thermal stressed situation and this result can be observed in the egg and in ovaries analyzed. *auts2 and dpysl5*

Despite the lack of significant differences we can observe a tendency for *auts2a*. The *auts2a* gene seems to be dysregulated in the ovary in opposition to the pattern observed in the egg. The expression of the *auts2a* gene seems to be less important in ovaries from thermally stressed mothers in comparison to ovaries from mother bred at the normal temperature. Contrary to *auts2a* and even if there are no statistical differences, we can note differences in *dpysl5* expressions in eggs as means ± standard deviation (SD) are not overlapping. The

expression of *dysl5* is less important in eggs from thermally stressed mothers than in eggs from mothers bred at the control conditions.

3.2 Post-larval phenotyping results

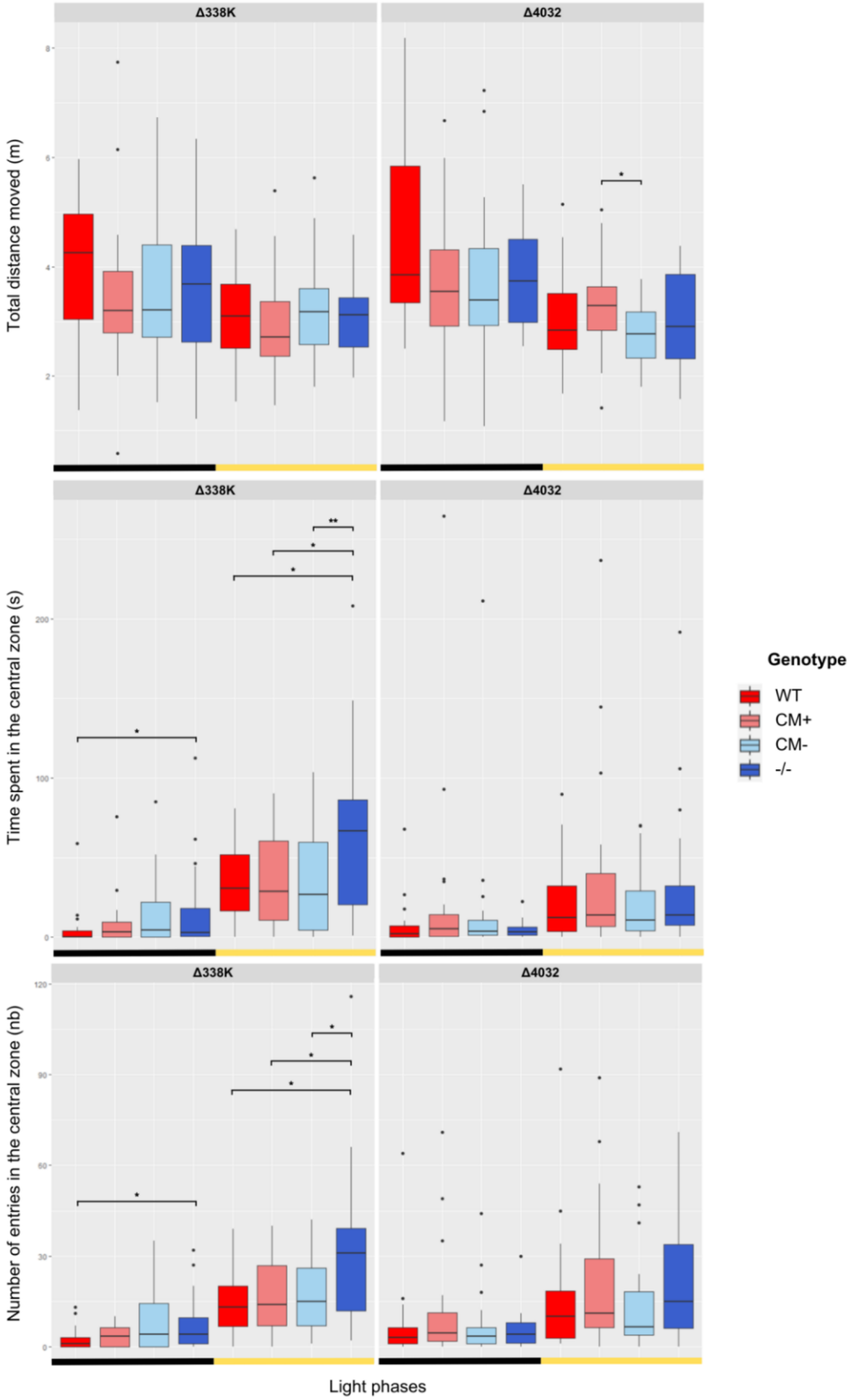

Figure 11: Response of auts2a KO medaka's offspring post-larvae to light stimuli .
*Two lines of auts2a KO medaka were video tracked for 10 minutes in darkness and 10 minutes light in a circular area with an artificial central zone. *P < 0.05 and **P < 0.01 indicate significant differences between genotypes.*

Total distance moved

Firstly, we can note that the genotype does not exert a significant effect on the total distance moved during the experiment in the case of the Δ338K line. In opposition, a significant difference is noticeable in the total distance moved between CM+ and CM- groups of the Δ4032 line.

Furthermore, we can also observe that for every genotype within each line, the mean distance traveled is consistently reduced during the light phase as compared to the dark phase. Hence, whatever the genotype and the line, it appears that post-larvae cover greater distance during the dark phase compared to the light phase.

Time spent in the central zone

Contrary to the Δ4032 line, significant noticeable disparities can be observed in the time spent in the central zone between Δ338K's genotypes within phases. In the case of the Δ338K line, significant differences appear during both light phases of the experiment, which contrast with the Δ4032 line where no statistically significant differences are observed across phases.

For the Δ338K line, it appears that homozygous mutant fish spend significantly more time in the central zone than wild-types during periods of darkness. Additionally, this outcome remains consistent even during the light phase. Consequently, whatever the light period, homozygous mutant fish demonstrate a consistent tendency to spend more time in the central zone than their wild-types counterparts.

Significant differences between genotypes are more numerous during the light phase. The time spent in the central zone exhibits significant variation between homozygous mutant fish and all other genotypes. As a result, it becomes evident that, during the light phase, homozygous mutant fish spend more time in the central zone of the area than their wild-type counterparts.

Moreover, we can also note that, across all lines and genotypes, the average time spent in the central zone is consistently higher during the light period compared to the dark phase.

Number of entries in the central zone

A similar trend to that seen in the time spent in the central zone appears when we investigate disparities in the number of entries in that same zone. Likewise, statistical differences are observed exclusively within the Δ338K line and contrasting with the Δ4032 line.

In the case of the Δ338K line, homozygous mutants display a higher propensity to enter the central zone of the area compared to wild-type during both dark and light periods. As for the previous results, significant disparities are more numerous during the light phase. In that second phase, we observed that CM+ and CM- are also less likely to enter in the central zone than homozygous mutant fish. Hence, homozygous mutant fish are more likely to enter in the central zone than wild-types whatever the light phase and than all other genotypes during the light phase.

Moreover, as for time spent in the central zone, the average number of entries in the central zone is always higher in the light phase than in darkness for each genotype of each line.

3.3 Social behavior experiment results.

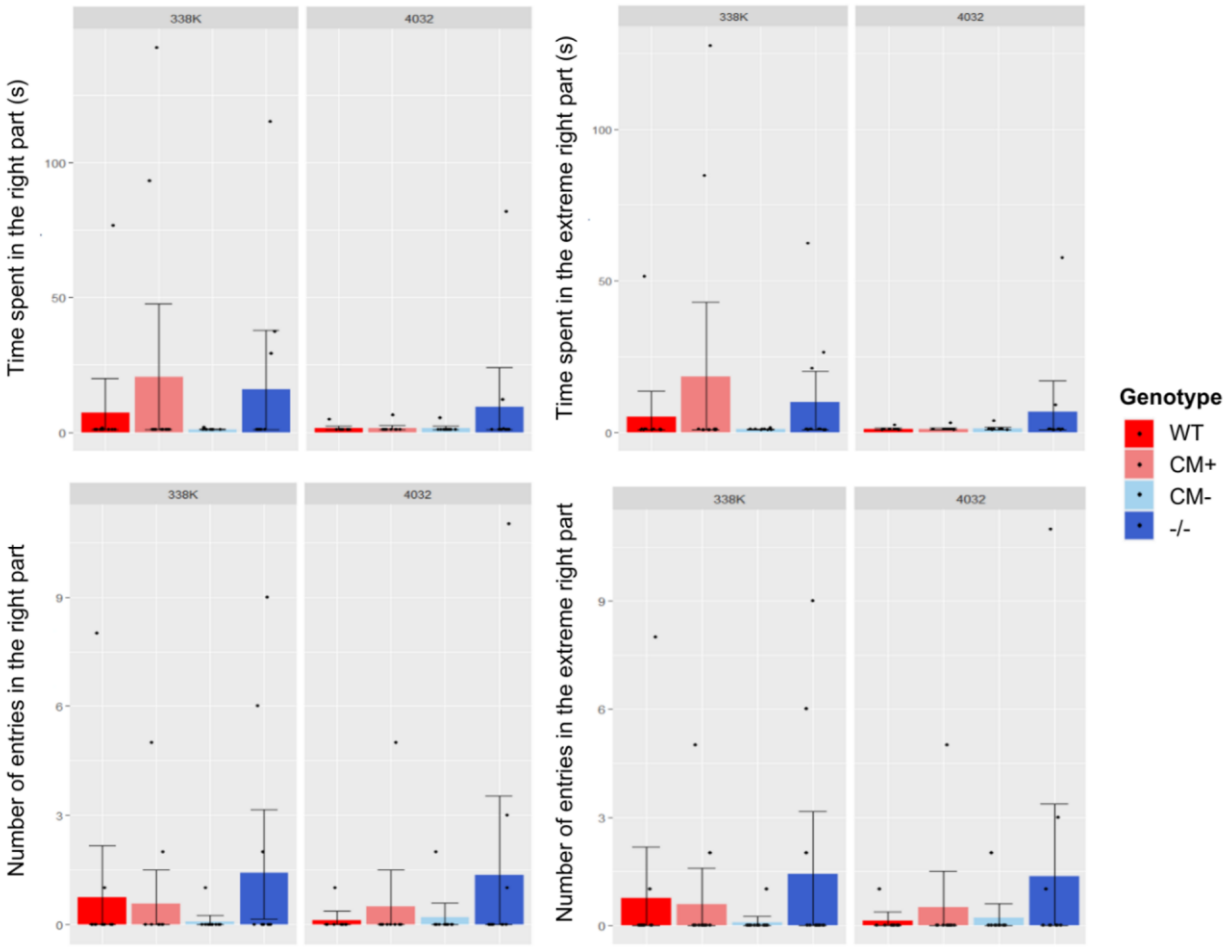

Figure 12: Response of auts2a KO medaka's offspring to social stimuli.
*Two lines of auts2a KO medaka were video tracked for 5 minutes in an aquarium with, in the left side, a social stimuli with an aquarium of 6 fish and, in the right part, an empty aquarium. The tracking period was preceded by a 10 min adaptation phase. *P < 0.05 and **P < 0.01 indicate significant differences between genotypes.*

Time spent in the right part and extreme right part

Statistically significant differences are absent in the time spent in the right part and in the extreme right part of the aquarium between genotypes. Similarly, significant disparities are not observable in the number of entries into both of the right parts of the aquarium. In both of the observed variables, means ± SD between genotypes are not overlapping and no distinct trend is apparent.

It is noticeable that, among the genotypes observed, there exists a substantial variability in behavior across individuals. This variability is evident across all genotypes within the Δ338K line, with an exception of CM-, which similarly holds true for the Δ4032 line.

In contrast to the Δ338K case, the homozygous mutant from the Δ4032 line appears to exhibit the most pronounced variability when compared to all other genotypes. Moreover in the Δ4032

line the maximum of the two observed variables is always displayed by an homozygous mutant.

4. Discussion

4.1 Thermal stress experiment

Aim and results

Our objective was to explore how thermal stress affects the expression of neurodevelopmental genes in eggs and ovaries of female medaka.

Regarding the outcomes of our experiment, it is apparent in medaka that thermal stress has an influence on the expression of neurodevelopmental genes in the egg and in the ovary. Out of the 4 genes investigated, 3 exhibit dysregulation in eggs: *arv1*, *dpysl5* and *plp2*.

Additionally, in contrast to *dpysl5*, *arv1* and *plp2* demonstrate up-regulation under thermal stress conditions. In ovary, the thermal stress affects the expression of only 2 out of the 4 examined genes: *arv1* and *plp2.* Moreover, there is a discernible trend for *auts2a* as well. We can note that both arv1 and plp2 exhibit up-regulation in ovary as in egg. The genes targeted through RT-qPCR are known as neurodevelopmental genes, associated with neural disorders and linked to cognitive disabilities.

The *auts2* gene, associated with multiple neurological disorders and involved in neurodevelopment has been described previously. The absence of significant differences in *auts2a* within eggs can be attributed to its natural low level of expression in this tissue. Maternal inherited *auts2a* has been identified in medaka's activated eggs, however the expression level at this stage is notably lower compared to after the zygotic genome activation (Merdrignac et al, 2022). Highlighting significant disparities between two expression levels can be challenging when working with limited RNA quantities.

The *dpysl5* gene (dihydropyrimidinase-like 5) belongs to the collapsing response mediator protein (CRMP) family, which plays a role in neurodevelopment. CRMP genes are recognized for their elevated expression in the developing brain and in adult brain areas that preserves neurogenesis. Although their functions in the adult brain remain poorly known, they are recognized for their substantial role in axon guidance signaling and neurite outgrowth. The lack of CRMP can lead to a notable rise in both proliferation and neurogenesis (Veyrac et al, 2011).

Knowledge about the functions of the ARV1 protein are scarce, however a deletion on the gene results in multiple development troubles. It has been shown that mutation on the *ARV1* gene resulted in severe neurodevelopmental delay and intractable onset seizure disorder (Palmer et al, 2016).

The role of the *plp2* gene in fish is still unknown but PLP2 polymorphism has been associated in humans with X-linked mental retardation (Zhang et al, 2007).

All genes that demonstrate dysregulation under thermal stress conditions are linked with neurodevelopmental issues and cognitive disorders. Consequently, we can deduce that maternal thermal stress in medaka affects the expression of maternal factors in both ovaries

and eggs, and potentially the phenotype of the offspring. To establish the link between gene regulation and phenotypic plasticity of the offspring, it could be interesting to implement morphological phenotyping or behavior analysis on the offspring. We can also imagine conducting phenotypic analyses on the embryonic mortality rate at various stages. This can allow us to link thermal stress with developmental issues and egg quality for this research fish model.

Comparison with rainbow trout

The aim was also to compare these results with outcomes found on rainbow trout in order to ascertain the generalizability of the conclusion to medaka.

All the results obtained in medaka eggs are consistent with previously observed results in rainbow trout, except for *auts2*. The genes *arv1*, *plp2* and *dpysl5* are dysregulated under thermal stress conditions in eggs in both studies. Furthermore, similar trends are observed for *arv1* and *plp2*, as they are up-regulated, and for *dpysl5*, which is down-regulated in both medaka and trout (Colson et al, 2019).

While these results in medaka align with those found on rainbow trout, a notable discrepancy arises in the auts2 expression in eggs. This difference could potentially be attributed to species-specific biological characteristics. Medaka, hailing from rice paddies, is used to temperatures ranging from 15°C to 28°C, in contrast to rainbow trout, which experiences temperature variations in a lower range.

In summary, it can be affirmed that a maternal thermal stress induces the dysregulation of neurodevelopmental gene expression in both species. This phenomenon in medaka might be correlated with impairment of cognitive responses, as evidenced in rainbow trout, through additional analyses.

Furthermore, these outcomes could potentially correspond with findings observed in diverse animal species. Evidence from studies on birds and fish has demonstrated that a maternal stress before fertilization triggers cognitive impairment in the offspring, compared to the offspring of non-stressed animals (Guibert et al, 2013. Eaton et al, 2015). Similar findings have also been observed in mammals, where maternal stress during pregnancy can elevate the risk for children to suffer emotional issues and development troubles (Zagron & Weinstock, 2006).

Setup issues

The fish used for the experiment underwent an initial 10-days habituation phase followed by a month of sampling period under the consistent temperature conditions (26°C and 32°C). However, subsequent to these initial 10 days and during the sampling phase, we began to note several issues that started to disrupt the smooth progression of the experiment.

Firstly, we observed a significant mortality rate among fish, particularly among the females within the aquariums. It's important to note that this substantial mortality affected fish from both aquariums regardless of the temperature condition. Despite the isolation of the room used for this setup and the water renewable each day, we hypothesized the presence of a bacteria that colonized both tanks, resulting in the loss of several fish, sometimes occurring overnight. These losses have affected our ability to reach the initial target sample of 600 eggs per condition, consequently extending the sampling period beyond our initially intended

timeframe. This might have impacted on the observed results and how we interpret the outcomes.

First, the extended sampling phase implies that all eggs were not collected at the same period of the experiment, then they didn't all originate from mothers exposed to the same duration to the thermal stress.

Secondly, the potential existence of bacteria in the setup introduces a bias into the results of the experiment. Depending on the inter-individual variation, fish can display varying degrees of sensibility to this factor. Although we did not observe a significant difference in the mortality rate between the two aquariums, we can hypothesize that fish subjected to thermal stress could be more sensitive to the stress induced by these bacteria.

Additionally, it's important to note that the unanticipated mortality rate had repercussions on the sampling strategy. Indeed, we were forced to reduce the number of extracted ovaries, which subsequently have an impact on the statistical power of our results.

Moreover, we observed a decline in the egg quality from the thermal stress mother over the sampling period. The majority of these eggs started to exhibit signs of unfertilized eggs and became susceptible to damage after having been collected. Consequently, the egg sampling became harder and good quality eggs became scarce. For these eggs, it was harder to identify and to separate the unfertilized egg from the empty ones.

Lastly, we observed that several females were not spawning. We did not observe significant disparities in this phenomenon between the two aquariums but eggs collected originate from a part of the female used in that experiment. This had an effect on the initially established sample plan. We needed to decrease the number of ovaries collected since our focus was on working with spawning females.

RNA extraction issues

We didn't not manage to extract RNA from every egg sample. After completing all the protocol steps, the RNA cheek that should be present at the bottom of the tube was not observable in each sample. Indeed, for some samples we failed to extract any RNA. This can be linked to the variable quality observed during the sampling period and especially because eggs were pooled by 100 without considering the collection date.

4.2 Post larval phenotyping

Aims

Initially, the goal was to examine the light stimulus response of offspring from *auts2a* mutant medaka, focusing on 2 lines associated with distinct mutations.

The primary objective was to analyze each line separately. In the Δ338K line, the aim was to explore the maternal effect of *auts2a* on the offspring behavior and to compare the resulting phenotype with the previously identified one. In the case of the Δ4032 line, the goal was to investigate offspring behavior and attempt to establish a phenotype linked or not to the effect of maternal contribution of auts2a.

The second objective was to compare the two investigated lines. The goal was to attempt to establish links between the observed outcomes and the mutation localization within the gene for each line.

Results for the Δ338K line

No discernible effects of the genotype and of maternal contribution of *auts2a* on post-larval activity were observed. There are no statistically significant differences in the total distance covered between genotypes across the 2 light phases of the experiment. The total distance traveled appears to remain consistent within genotypes, and particularly during the light phase which exhibits lower inter-genotypic variability.

As developed in the results part, we can observe that the mean total distance moved is consistently lower during the light phase compared to the dark phase for each genotype. Furthermore, as described previously, the total distance traveled is recognized as an indicator of fish activity, a factor known to be correlated with the stress experienced by the subjects. Consequently, we can observe that post-larvae medaka are more likely to be stressed during the dark phase as opposed to the light phase.

In this study, we created an artificial central tracking area to explore thigmotactic behavior by firstly analyzing the time spent within this central zone. As described in the context part, thigmotactic behavior is recognized as linked with stress-inducing situations and is a prevalent behavior in adult medaka.

Firstly, a behavioral switch between the two light phases is evident with the average time spent in the central zone consistently higher during the light phase compared to the dark phase. As a result and linked with previous results, we can observe that medaka post-larvae exhibit thigmotactic behavior linked with stress-inducing situations, notably darkness.

Secondly, statistically significant disparities appear across genotypes in the time spent in the central zone. During the period of darkness, when subjects are less inclined to explore the central region, it becomes noticeable that homozygous mutant fish have a higher tendency to remain in the central area compared to their wild-type counterparts. During the light period, homozygous mutant fish appear to exhibit a lower degree of thigmotactic behavior compared to all other genotypes. As a result, we can observe that an homozygous auts2a mutation in medaka leads to a reduction in anxiety-like behavior, as evidenced by a decreased level of thigmotactic behavior. Moreover, these disparities seem to be amplified in situations without any stress stimuli.

Finally, we investigate the number of entries in the central zone in order to enhance the analysis of thigmotactic behavior and to ascertain whether the time spent in the central zone was connected to exploratory behavior of fish within the total tracking area. Indeed the same trend is observed in the time spent in the central zone and in the number of entries in that zone regarding the significant disparities. Consequently, we observed that mutant homozygous medaka for *auts2a* are more likely to explore the area than their wild-type counterparts.

To conclude, an *auts2a* mutation in medaka triggers a reduction in the anxiety-like behavior and an increase in the exploratory behavior. Moreover, these exploratory tendencies cannot be attributed to higher activity levels, as activity is described as consistent between genotypes during both light phases of this experiment.

Comparison with previous outcomes on the Δ338K line

As in our work, it has been previously shown that homozygous adult medaka with this Δ338K mutation display reduced anxiety-like behavior than wild-types. However, disparities between CM- and all other genotypes with auts2a maternal contribution have been highlighted. Contrary to our work in medaka post-larvae, in the adult medaka, we observe an effect of the absence of *auts2a* maternal contribution. Previous outcomes described the *auts2a* maternal contribution alone responsible for a better recognition of the environment and learning abilities.

We can postulate that the absence of an effect from the maternal contribution of *auts2a* (associated with this Δ338K mutation) in our study might be attributed to the developmental stage of the analyzed subjects. It is recognized that *aust2* is involved in gene expression regulation and activation and the presence or the absence of its maternal factor within the egg can potentially initiate long-term effects on the progeny.

It is known that the *aust2a* maternal factors are involved in the early nervous system development which is associated with major long-term consequences on the offspring's behavior. In the early stage of development, such as post-larvae, only the distinction between homozygous mutants and wild-types is discernible. As fish continue to develop, the effect of *au2a*'s maternal contribution can appear in offspring behavior.

Results for the Δ4032 line

No statistically significant differences have been noted among all genotypes in each light phase and for each analyzed variable, except for the total distance moved where a distinction is observed between CM+ and CM-. Based on these results, drawing conclusions about the maternal effect of auts2a's maternal contribution is complex, and elaborating meaningful outcomes is difficult.

However, we can observe that across all genotypes, the average total distance moved is always lower during the light period compared to the dark period which aligns with outcomes on the previous line. Consequently, that confirms that post-larvae medaka are more likely to be stressed during the dark phase as opposed to the light phase as their activity levels are higher.

Additionally, we also observe that post-larvae from this line seem to spend more time in the central zone during the light period than in darkness. According to the average number of entries in the central area, post-larvae medaka from this line seems to display thigmotactic behavior which aligns with findings on the previous line. Consequently, that confirms that medaka post-larvae exhibit thigmotactic behavior linked with stress-inducing situations, notably darkness.

Comparison of the Δ338K line and the Δ4032 line

As previously observed, the 2 investigated lines exhibit similarities that amplify the significance of the observed disparities. Firstly, thigmotactic

We have seen it previously, the 2 lines investigated display similarities which strengthen the power of the observed disparities. Indeed, thigmotactic behavior coupled with consistent activity was observed in both lines. It is also important to highlight that statistical analyses were also conducted to compare the wild-type individuals between both lines for each phase,

employing the same methods as previously described, and no significant differences were detected. Therefore, all these findings contribute to the robustness of the differences observed between the 2 lines.

As the significant result observed for the Δ4032 line is complex to interpret, our attention will be directed towards investigating the thigmotactic behavior, which is characterized by the time spent in the central area and the number of entries in that area. In opposition to the Δ4032 line, in the Δ338K line, we can observe an effect of an homozygous mutation of *auts2a* on the post-larvae behavior. Consequently, we can conclude that a deletion of almost 90% of the *auts2a* gene in the N-terminus part in the medaka triggers a reduced anxiety-like behavior at the post-larval stage. Moreover, we can add that a deletion of almost 4,000 bp in the C-terminus part of the gene does not have an effect on the anxiety-like behavior at the post-larval stage.

The deletion associated with the Δ338K line starts at the exon 2 to extends to exon 7. This deletion notably affects a substantial proportion of the gene, including consequences with identified functions. The proline-rich domain, identified within exon 7 and 8, is directly affected. Additionally, this deletion alters the structure of the full-length protein, which possesses numerous documented functions.

The AUTS2 protein is known as a transcription factor that interacts with transcription regulators. It plays a key role in a complex that regulates the activation or the repression of genes within the central nervous system. Within this complex named PRC1, AUTS2 is linked with the recruitment of 2 other proteins (P300 and CK2) with identified function in the gene expression regulation (Gao et al. 2014). We can hypothesize that a deletion covering 90% of the gene could significatively influence its functions, particularly when it involves the removal of a proline-rich domain linked to mRNA-binding function. Moreover, another study has highlighted the importance of the HX motif in this recruitment process which can be impacted by a structure modification linked with the Δ338K deletion (Liu et al, 2021).

Several studies have exposed the disparities between the 2 main isoforms of the AUTS2 protein (FL-AUTS2 and var2-AUTS2, Figure 2). It has been suggested that the N-terminus part of the protein might be responsible for transcription repression while the C-terminus part can be responsible for the expression activation. It has also been shown that, during neural differentiation, there is a shift in isoform expression with a FL-AUTS2 isoform expressed in undifferentiated cells and subsequently being supplanted by a shorter isoform during the differentiation process. Moreover, evidence has emerged indicating that the only presence of the short isoform leads to an early neural differentiation and that the absence of the full-length isoform might explain the observed phenotype linked with an accelerated neural differentiation (Monderer-Rothkoff et al, 2019).

Finally, it has been shown that with a mutation involving the proline rich domain affected by our Δ338K deletion, we observe a significantly lower proportion of lamellipodium. As a result, it means that AUTS2 is involved in the neuronal migration with the PR2 domain localized on the exon 7 and 8 of the gene (Figure 2) (Kei Hori et al. 2014).

Role of the N-terminus part of the gene is described through the identified function of the full-lenght AUTS2 protein. Variations in behavior within the 338K line was expected as it is associated with a large deletion of the gene. Moreover, the key role of the delated part has previously been detailed and the effect of that mutation on the medaka behavior has already

been highlighted. On the other hand, the deletion of the Δ4032 line affects the auts2 domain of the protein which is a poorly documented domain which seems to have no effect on the post-larvae behavior.

Setup issues

One of the main issues encountered during the experiment's protocol was the process of selecting larvae for the tracking experiment from the petri dish to the plate. To ensure an adequate number of larvae for the test, we intentionally bred more larvae than required, necessitating a subsequent selection process. The selection was carried out during the plate loading using a highly randomized approach in order to avoid any experimental bias.

Additionally, the effects of the mutation have introduced disruptions in the seamless progression of the experiment. Firstly, there were variations in densities within the aquariums linked with differences in survival rates among genotypes within each line. Despite our efforts to maintain similar densities, this factor may have influenced the observed results. Secondly, the developmental delay described in previous study in larvae with the Δ338K mutation has introduced a variation in size among genotypes, potentially influencing the results, particularly in terms of the total distance moved.

Finally, we chose to implement a 20-minute habituation phase in darkness based on previous published findings. However, upon examining our results, it becomes apparent that the dark period acted as a stress-inducing situation for the fish. Optimal condition to establish a basal activity in our subject might be to conduct the habituation phase under light exposure.

4.3 Social behavior experiment

Aims and results

The main objective of the experiment was to explore the social behavior of offspring from auts2a mutant medaka, focusing on 2 lines linked to different mutations. Initially, the aim was to assess the effect of the maternal contribution of auts2a on the social behavior of the offspring within both lines. Subsequently, the objective was to examine the impact of the auts2 mutation's location on the response to social stimuli.

No notable significant differences were observed between genotypes within each line. The presence of intra-individual variability introduces challenges in the results interpretation. However, we note a trend within the Δ4032 line, where homozygous mutant fish appear to spend more time in the right part of the aquarium. While definite conclusions cannot be drawn, it is plausible to hypothesize that homozygous mutant medaka with the Δ4032 mutation might exhibit reduced attraction to social stimuli compared to the other analyzed genotypes. Validating this hypothesis should involve conducting additional social tests and increasing the number of subjects. Moreover, the lack of significant disparities exposed in our results contrasts with findings on other species. As described previously, it has been shown that Auts2 KO mice can exhibit social deficits.

Experimental issues

First, the experiment is time-consuming due to the substantial time required for setting up each test and conducting the test itself. This time constraint triggers challenges to increase the number of analyzed subjects. Moreover, the video processing phase is also quite time-consuming, particularly when configuring the tracking area between each test due to minor variations in the videos.

Secondly, as previously outlined, our decision was to initiate the tracking phase immediately after the habituation phase, coinciding with the fish's initial entry into the ELZ. However, we have encountered tests where certain fish consistently remain in the right section of the aquarium, rendering them unanalyzable. Additionally, we have identified reflections on both sides of the aquarium (ERZ and ELZ) that might have influenced fish behavior, potentially creating a social stimulus in the right area of the aquarium through their own reflection.

Lastly, it's important to note that this experimental setup induced stress in the fish. The new environment combined with isolation during the habituation phase could have influenced the observed outcomes. Furthermore, upon removing the separation, we observed flight behavior followed by freezing sequences in some fish. These stress-induced behavior might introduce a bias in our results.

Conclusion

My internship at the INRAE FPGL had multiple objectives and was a component of a larger research project.

The primary goal was to explore how the absence of *auts2a* maternal contribution, linked to distinct mutations, impacts medaka behavior. To that end, we examined post-larvae responses to light stimuli and adult reactions to social stimuli. In opposition to previous findings on the Δ338K deletion, we couldn't confirm that the lack of maternal *auts2*a contribution leads to reduced anxiety-like behavior. We didn't observe any maternal effect of *auts2* for both mutation and for both experiments. This may be explained by the stage disparities between analyzed fish in the post-larval phenotyping compared to the fish used in previous experiments or by the lack of appropriateness or quality of our social experiment.

The second objective was to investigate behavioral phenotypes of these two medaka lines in order to conduct a functional analysis of the AUTS2 protein according to the deletion localization. Notably, we managed to demonstrate that homozygous mutants in the Δ338K line displayed lower anxiety-like behavior than wild-type, contrary to the Δ4032 line. Considering AUTS2 structure findings previously described, we can hypothesize that the auts2a Δ338K deletion affects protein function, impacting the post-larval phenotypic plasticity compared to the Δ4032 deletion which seems to have no effect on the post-larval behavior.

Lastly, we aimed to assess the effect of a thermal stress on the neurodevelopmental gene expression as previously done in rainbow trout. We managed to demonstrate that thermal stress triggers impairment of gene expression in medaka's egg and ovaries and especially for genes involved in the development of the neural system.

As a global conclusion, the overarching goal of the AUTS2 project is to understand the role of *auts2a* in medaka's neurodevelopment and behavior, with a specific focus on the effects of its maternal contribution. This subject has broader implications, including the exploration of

intergenerational stress effects, an increasingly pertinent concern for sectors like aquaculture, particularly with the current context of global warming.

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