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Rôle de l'expression maternelle du gène *auts2a* dans le déterminisme du comportement chez la larve de médaka *(Oryzias latipes)*

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Soutenu à Rennes le 11 septembre 2024

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Contents

Glossary

Aa: Amino acid

ASD: Autism Spectrum Disorder

AUTS2: Transcription activator and development regulator gene

CV: Coefficient of variation, a measure of the dispersion of the data in relation to the mean, expressed as a percentage.

Dpf: Day post-fertilization

Dph: Day post-hatching

FPGL: Fish Physiology and Genomics Laboratory

GLMM: Generalized Linear Mixed Model

HGNC: HUGO Gene Nomenclature Committee

HME +: Heterozygous mutant with maternal expression of the *auts2a* gene

HME -: Heterozygous mutant without the maternal expression of the *auts2a* gene.

L1: First phase of light

L2: Second phase of light

Lux: Unit of light measurement

Mutant: Homozygous mutant of the *auts2a* gene

O1: First phase of darkness

O2: Second phase of darkness

SOBs: Sex, Oogenesis and Behaviors team at the Fish Physiology and Genomics Laboratory

WT: Wild type

In this study, the nomenclature used to designate the *AUTS2* gene and its homologues in different species was chosen to reflect conventions established in the scientific literature and to ensure optimal clarity.

For the human gene, we use the italic *AUTS2*, in accordance with the convention of the HUGO Gene Nomenclature Committee (HGNC) that gene names should be italicized in *Homo sapiens*. In mice, the spelling *Auts2* (in italics) is adopted, with an initial capital letter to indicate that it is a gene in a non-human mammalian model, in this case *Mus musculus*. For the teleost fish, the gene is written in lower case and italics: *auts2*, following the nomenclature convention. For the protein, the nomenclature follows similar rules, but without the use of italics. When reference is made to the gene or protein without specifying a particular species, the terms *AUTS2* (for the gene) or AUTS2 (for the protein) are used, in italics or not, respectively.

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1. Introduction

1.1 The *AUTS2* gene: Role and impact in neurodevelopmental disorders

Discovery and name of the *AUTS2* gene

In recent years, advances in genetics have made it possible to identify high-risk genes associated with certain neurodevelopmental diseases, such as Autism Spectrum Disorders (ASD) (Pang et al. 2021). Among them, the *AUTS2* gene is of particular interest. This gene was first discovered in monozygotic twins with autism in 2002 (Sultana et al. 2002) and named for 'autism susceptibility candidate 2' to recently become 'activator of transcription and developmental regulator' according to the HGNC.

The *AUTS2* syndrome

Indeed, *AUTS2* has been associated with a syndromic form of intellectual disability known as '*AUTS2* syndrome'. Individuals with this syndrome present a variable phenotype, including ASD, schizophrenia, epilepsy, neurological and developmental disorders that affect the way they interact, communicate and learn (Hori, Hoshino 2017; World Health Organization 2019). These characteristics are also associated with distinctive physical features, such as microcephaly, hypotonia, short stature and facial dysmorphia. *AUTS2* syndrome is thus characterized by a severity score based on those criteria (Beunders et al. 2013). The correspondence between the localization of *AUTS2* gene mutation and the phenotype observed is well documented. (Hori, Shimaoka, Hoshino 2021). For example, studies have shown that patients with deletions of C-terminal regions of *AUTS2* have severe phenotypes of *AUTS2* syndrome (Beunders et al. 2013).

Expression and role of *AUTS2* in the brain

The *AUTS2* gene encodes a 1259 amino acid (aa) protein in humans and *Auts2* encodes a 1261 aa protein in mice (*Mus musculus*). Numerous isoforms are generated by alternative transcription sites and alternative splicing (Beunders et al. 2013). These transcripts are notably present in the embryonic brain, with differential expression depending on the stage of development (Pang et al. 2021). The AUTS2 protein acts both in the cytoplasm and in the nucleus of cells. During brain development, AUTS2 participates in the differentiation of neurons via epigenetic regulation of transcription by acting on histones, regulates the balance between excitatory and inhibitory synapses (Hori, Shimaoka, Hoshino 2021), and participates in the elongation and migration of neurons by modulating the organization of the cytoskeleton (Pang et al. 2021; Clément 2023). This multidimensional role makes *AUTS2* a central subject of study in the understanding of neurodevelopmental disorders.

1.2 Impact of maternal stress on *auts2* expression

It has been shown in rainbow trout (*Oncorhynchus mykiss*) that heat stress applied to female spawners leads to deregulation of certain maternal factors. Maternal factors represent all the maternal messenger RNAs and proteins deposited in the egg during oocyte maturation. They are responsible for the early structuring and regulation of embryo gene expression by conditioning the activation of the zygotic genome (Harvey et al. 2013). These deregulated maternal factors include those associated with the *auts2* gene in the oocyte. This deregulation was linked to behavioral problems in the offspring, in particular inhibition of locomotor responses associated to fear when the offspring is exposed to a new environment (Colson et al. 2019).

1.3 Development of the '*auts2*' project

The discovery of maternal impacts on the behavior of the offspring associated with this gene by the Sex, Oogenesis and Behaviors (SOBs) team at the Fish Physiology and Genomics Laboratory (FPGL), led to the development of the '*auts2*' project at the FPGL. The SOBs teamwork focuses on the environmental and transgenerational determinism of behavior. The aim of this project is thus to understand the mechanisms underlying the involvement of the *auts2* gene in the behavior and quality of brain development of offspring. This project is being carried out on the medaka (*Oryzias latipes*).

1.4 The medaka (*Oryzias latipes*): A model of interest for the study of *auts2*

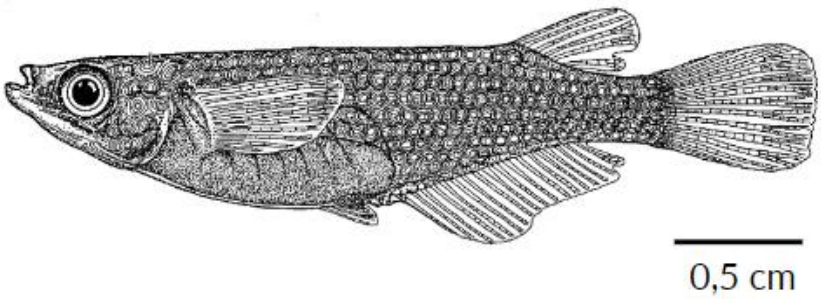


Figure 1: Graphical representation of an adult male medaka, (Iwamatsu, 2004).

Technical advantages

The medaka (*Oryzias latipes*) (Figure 1) is a species of teleost belonging to the *Adrianichthyidae* family and the *Oryzias* genus. It is a fish measuring a few centimeters that lives in fresh and brackish water and is native to India and South-East Asia. It is a reference species with many technical and genetic advantages (Iwamatsu 2004). It is very easy to maintain in an aquarium. Moreover, medaka spawning can be regulated by an artificial photoperiod of 16 hours of light and 8 hours of darkness (breeding photoperiod) and an optimum temperature (26°C) (Koger, Teh, Hinton 1999). Under these conditions, females lay around 30 eggs a day, which are fertilized externally. The transparent eggs are carried by the female in the form of a cluster on the anal fin until the next spawning. The eggs hatch in around ten days at 26°C (Iwamatsu 2004). The species also displays sexual dimorphism in its dorsal and anal fins. From a genetic point of view, the medaka has the advantage of being sexually determined by a gene called dmrt1, which is not the case for zebrafish (*Danio reiro*). The fact that it can be maintained in experimental facilities, the regularity of its spawning, the quantity of eggs produced, the speed of their development, the presence of sexual dimorphism and gene are all technical advantages of the species for its use in research.

auts2 gene in Medaka (*Oryzias latipes*)

The use of fish models for studying ASD represents a novel experimental approach aimed at complementing and enhancing current paradigms in rodents. This approach is based on the evolutionarily conserved nature of social behavior and its molecular pathways (Stewart et al. 2014). The choice of the medaka for this project is justified by the presence of a single copy of the *auts2* gene, unlike the zebrafish which has two copies (*auts2a* and *auts2b*). This feature simplifies the interpretation of the results by avoiding the complications associated with functional redundancy and paralog regulation. The absence of additional copies in medaka allows us to focus on the specific effects of *auts2a*, providing a clearer analysis of the phenotypes associated with the gene mutation.

1.5 Line and genotypes studied in the *auts2* project

Several mutant lines have been created to study the influence of *auts2a* gene mutations and their maternal expression. Among these lines is the Δ338K line, which includes a deletion of 338,000 base pairs corresponding to the deletion of 90% of the gene. The present study was carried out exclusively on this line. The genotypes of this line are detailed in Figure 2.

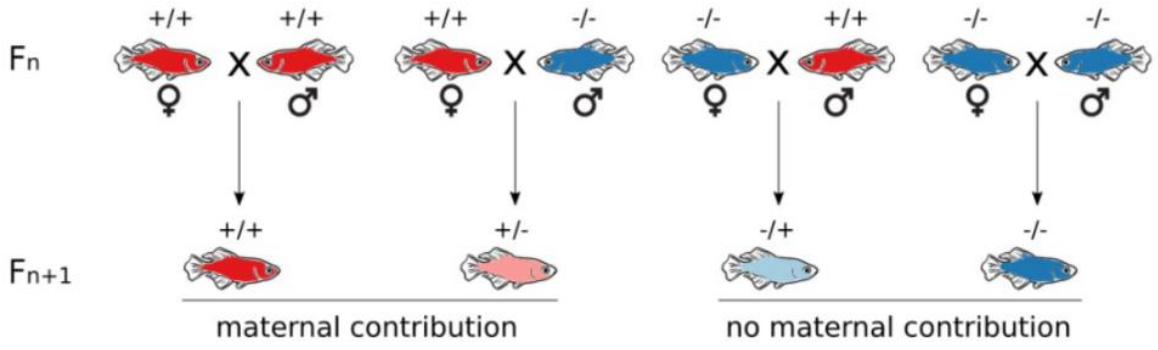


Figure 2: Genotypes studied for *auts2* project at the FPGL (Clément, 2023)

The Fn+1 generation thus has four genotypes. The wild-type (+/+) one, referred to as WT in the rest of the report, are descended from homozygous wild-type parents. The heterozygous with maternal expression of the *auts2a* gene (+/-) one, referred to as 'HME+', are descended from WT mothers and homozygous mutant fathers for the *auts2a* gene. The heterozygous without maternal expression of the *auts2a* gene (-/+), referred to as 'HME-', are descended from WT fathers and homozygous mutant mothers for the *auts2a* gene. Finally, homozygous mutant (-/-), referred to as Mutant, are descended from homozygous mutant parents for the *auts2a* gene. By comparing these genotypes, we can distinguish the specific contributions of maternal expression of the *auts2a* gene from its total absence, providing essential insights into the underlying mechanisms, its role and its impact on the development and phenotype of individuals.

1.6 Preliminary results of the *auts2* project

As part of the project, it was demonstrated that the absence of maternal expression of the *auts2a* gene in the Δ338k line (HME- and Mutant genotypes) resulted in several notable effects. Fish exhibited significantly smaller body and head sizes compared to individuals of the WT and HME+ genotypes. Additionally, a reduction in embryonic survival and poorer recognition of their environment were also observed (Clément, 2023).

Behaviors reflecting anxiety in this line were also affected. The new environment test was carried out on adult fish (3 months old). This test consists of placing individuals in a new environment, recognized as a stressful situation in many vertebrates including fish (Kittilsen et al. 2009) and associated with stereotyped behaviors reflecting anxiety. These behaviors include diving or geotaxis: an anxious fish positions itself at the bottom of the aquarium (Maximino et al. 2010). In adult fish lacking maternal expression of the *auts2a* gene, geotaxis is significantly less expressed than in fish with maternal expression of the gene (Clément 2023). This lack of caution may be interpreted as a 'risk-taking' behavior (Thomson, Watts, Pottinger 2011) reflecting lower anxiety in these genotypes.

In larvae, thigmotaxis, or the tendency of individuals to move closer to the walls of their environment, is modified. Thigmotaxis is also a stereotyped behavior reflecting anxiety (Richendrfer et al. 2012; Schnörr et al. 2012), the measurement of which is widely used as an indicator of anxiety in clinical studies in rodents (Schnörr et al., 2012), humans (Kallai et al. 2007), zebrafish (Richendrfer et al., 2012), and medaka (Lucon-Xiccato et al., 2022). In fact, preliminary tests seem to suggest that 21-days post-fertilization (dpf) larvae of the Mutant genotype display less thigmotaxis behavior than larvae of the WT genotype, suggesting a difference in anxiety in these larvae (Trillaud 2023).

Those differences in behavior reflecting anxiety observed in medakas lacking maternal expression of the *auts2a* gene are consistent with the results of the study that gave rise to the project as a diminution of fear response had been observed in the offspring of mothers stressed during oogenesis, presenting deregulations of the *auts2a* gene in the oocyte (Colson et al. 2019).

1.7 Aims of the internship

The main objective of this internship was to explore the impact of maternal expression of the *auts2a* gene on the behavior of medaka larvae at a larval stage that had not previously been studied in the project. To this end, two sub-objectives were defined:

1. To develop a rigorous experimental protocol for measuring reactions to a stressful event, which would serve as the methodological foundation for the behavioral analysis in the second part of the study.

2. To apply this protocol on the four genotypes studied.

The behavioral study focused on the reaction of the larvae to stress induced by light, with the hypothesis that individuals lacking maternal expression of the *auts2a* gene would show fewer behaviors reflecting anxiety than individuals possessing maternal expression of the gene, in line with the preliminary results obtained. This approach is well established in zebrafish (Emran, Rihel, Dowling 2008; MacPhail et al. 2009; Colwill, Creton 2011a; Vignet et al. 2013; Bai et al. 2016; Haigis et al. 2022), but very little in medaka (Le Bihanic et al. 2014; Chiffre et al. 2016; Dasmahapatra, Carty, Khan 2017).

2. Materials and methods

The tests were carried out in accordance with the principles of the use of laboratory animals and in compliance with French and European regulations on animal welfare (European Directive 2010/63/EU). They have been approved by the Committee for Ethics in Animal Experimentation of Rennes (CEAER) and the French Ministry of Research and Innovation under the approval number APAFIS # 32290-2022010611342063.

2.1 Breeding and reproduction of medakas (*Oryzias latipes*)

Mature Japanese medaka were reared and maintained in the laboratory's Collective Scientific Facility (CSF) in a closed circuit in 3-litre aquariums, with a ratio of 3 males for 5 females, and continuous water renewal. The temperature was maintained at 26°C with a light exposure of 300 lux for 15h45, followed by a dark period of 8h15 (reproduction photoperiod). The fish were fed 4 times a day on weekdays and 2 times a day at weekends with Gemma Wean feed (Skretting France: Crude protein 62%, Crude fat 14%, Crude ash 8%, Crude fiber 0. 2%, Phosphorus 1.1%, Calcium 1.5%, Sodium 0.6%, Vitamins A: 15000 IU/kg, Vitamins D3 1800 IU/kg, Iron 100 mg/kg, Iodine 5.1 mg/kg, Copper 15 mg/kg, Manganese 36 mg/kg, Zinc 100 mg/kg), 0.3 (350-500 µm).

2.2 Behavioral phenotyping

Egg collecting and selection of larvae

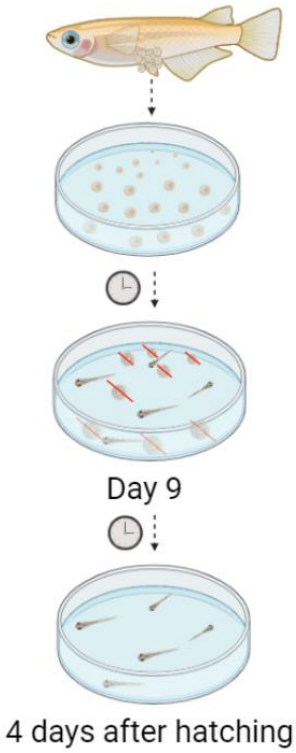


Figure 3: Egg collecting and larvae selection. Created with Biorender.com

Egg sampling was carried out at 3pm in the CSF, 7 hours after switching on the light, four times a week. The egg clusters were removed by hand and around one hundred eggs per genotype were collected each time. The collected eggs were placed in petri dishes containing Yamamoto embryo rearing medium (water, 0.1% NaCl, 0.003% KCl, 0.004% CaCl2, 0.016% MgSO4 and a few drops of methylene blue dye) (Figure 3). They were then individualized and unfertilized or dead eggs were removed. Petri dishes were placed in an incubator at 26°C and exposed to natural photoperiod for 13 days. The medium was changed once a day, except at weekends. Eggs that had died during development were also removed once a day.

To ensure uniformity in developmental stage, behavioral phenotyping was conducted on larvae at 4 days post-hatching (dph), a stage commonly used in studies conducted on medaka larvae (Le Bihanic et al. 2014; Chiffre et al. 2016; Dasmahapatra, Carty, Khan 2017). We also ensured that the larvae used had hatched at 9 dpf (Figure 3) as described in literature (Iwamatsu 2004). Only larvae without swimming defects were selected for the tests.

Experimental device: the Zebrabox (Viewpoint, France)

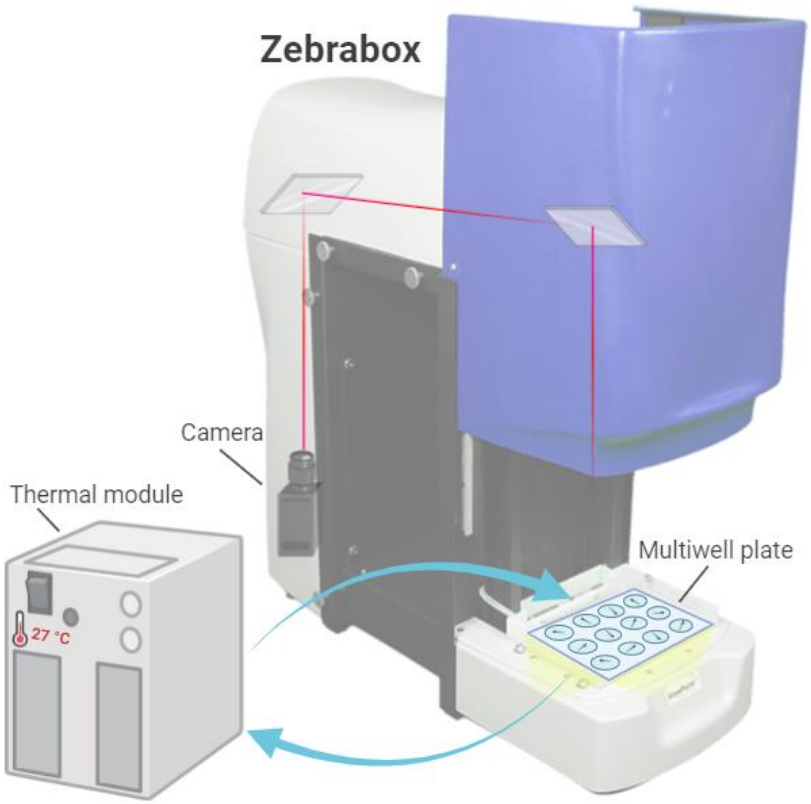


Figure 4: Details of the experimental device: the Zebrabox (Viewpoint, France). Created with Biorender.com

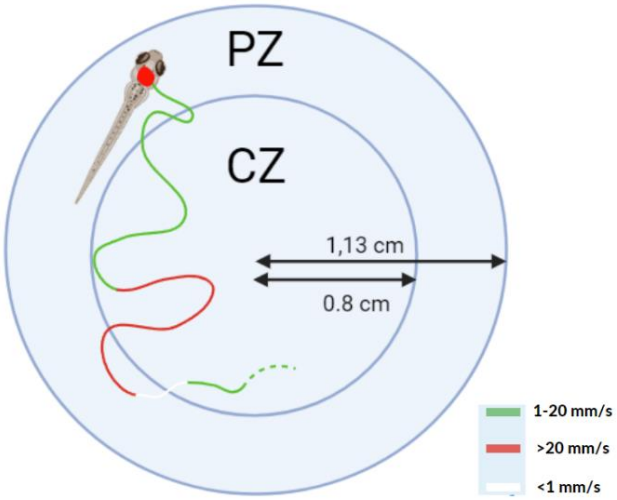
Behavioral observations were conducted using the Zebrabox (Viewpoint, France) device (Figure 4). It is an observation chamber with an infrared camera designed to analyze the behavior of model fish larvae and embryos. It is combined with Zebralab tracking and activity measurement software, which can be used to adjust all the detection parameters and the experimental protocol. The software generates a table of data for each experimental session. This table contains several information with numerous variables describing the activity of the individuals (distance travelled at a given speed threshold, time spent at this threshold, count of entries at this threshold, etc.). The Zebrabox also makes it possible to track several individuals at the same time using multi-well plates (Figure 4). The associated thermal module, represented in Figure 4, regulates the temperature and was set to 27°C.

Experimental setup and parameters entered in the Zebralab software

The 4 dph larvae were monitored in 12-well plates (Costar, Corning, USA), each filled with 1.14 ml of Yamamoto medium previously kept in the incubator. These plates were chosen because they offer sufficient space for the larvae to move freely, allowing differences in positioning to be observed, unlike plates with smaller wells as described in the literature (Padilla et al. 2011). To avoid experimental bias, several precautions were taken: only one individual was placed per well, and the whole device was placed in an isolated room.

All observations were made using Zebralab software, associated with the Zebrabox (Viewpoint, France). In the software, 12 arenas corresponding to the 12 wells with a diameter of 2.26 cm were created. For each well, two monitoring zones of equal area were defined: a central zone (CZ) of 1.6 cm in diameter and a peripheral zone (PZ) (Figure 5). This balanced distribution of zones avoids statistical bias linked to the positioning of the larvae, as used in behavioral tests with zebrafish (Colwill, Creton 2011a)

The detection parameters used were adapted from studies conducted on larval zebrafish using the Zebrabox system (Viewpoint, France) as detailed by Kristofco et al. (2016). Individuals were tracked from their center of gravity (Figure 5). The detection threshold was set to 180, and the animal color was specified as transparent. Motion thresholds were defined as inactivity for speeds less than 1 mm/s, cruising for speeds between 1 and 20 mm/s, and acceleration for speeds greater than 20 mm/s (Figure 5).

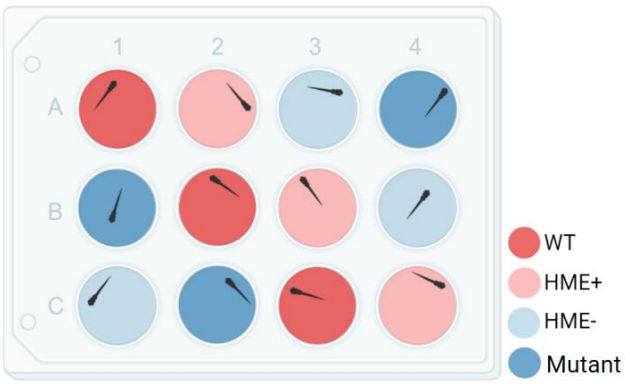


Data compilation intervals were set to 5 minutes for the test conducted on the WT genotype exclusively and 1 minute for the tests conducted on the four genotypes. For the light phase, an intensity of 300 lux was selected to match that of the experimental facilities, while the dark phase was maintained at 0 lux. To minimize the loss of larval detection, the background refresh rate was set at 1 minute. In addition, to reduce background noise, the minimum horizontal size of the object to be detected was set to 4 pixels.

Figure 5: Well zoning and larva tracking (CZ: Central Zone, PZ: Peripheral Zone) Created with Biorender.com

Experiments protocol

For all the test conducted during the experimental hours, the larvae were taken arbitrarily from the petri dishes in the incubator and transferred to a multi-well plate (Costar, USA) (Figure 3). The plates were then transferred to the Zebrabox (Viewpoint, France) (Figure 3) as gently as possible. The lid of the device was closed, and an additional layer of fabric was placed over the device to ensure that no external light could enter. The recording session was then started, with varying durations of light and darkness exposure depending on the tests conducted.



For the tests involving the four genotypes, between each experimental session, the plate plan (Figure 6) was modified by shifting the position of the four genotypes one position to the right to avoid any positioning bias. The light protocol (Figure 12), defined after preliminary tests to design it (presented in the Results section) was then applied. These tests were carried out over 7 days, with between 2 and 4 multi-well plates tested per day.

Figure 6: Example of a plate plan. Created with Biorender.com

Exclusion Criteria and Data Selection

For the tests conducted on WT genotype exclusively, no exclusion criteria were applied.

For the tests conducted on the four genotypes, individuals exhibiting signal loss (more than 15 seconds of signal loss within a minute) were excluded from the analysis. In total, this accounted for 27 individuals (WT: n = 2, HME+: n = 6, HME-: n = 10, Mutant: n = 9). Consequently, data from 260 larvae were retained (WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62).

Post-processing data and studied variables

We calculated several variables using the data provided by the software, such as Total distance traveled, Time spent in motion as well as Time spent in the peripheral zone. We also used the variables directly calculated by the software such as the Counts of inactivity (Inact) and high activity (Larct) entries. Those variables are commonly used to characterize the activity and stress of fish larvae (Chiffre et al. 2016; Haigis et al. 2022; Lucon-Xiccato et al. 2020).

Statistical analyzes

Statistical analyzes were performed using RStudio Statistical Software version 4.3.1 (The R Foundation for Statistical Computing, Austria). The additional packages used beyond those initially included in the software were: "lme4" for creating mixed models, "car" to calculate ANOVA tables for the created models, and "emmeans" for conducting post-hoc tests. All graphs were created using the "ggplot2" package.

The first step of the statistical analyzes involved evaluating the distribution of the data. For each counting variable, a Generalized Linear Mixed Models (GLMM) with a Poisson distribution was used. The other variables were also analyzed thru a GLMM but with a Gamma distribution. Genotype and light phase type were considered as fixed explanatory factors, while individuals (defined by the day of testing, time of testing, and their position in the plate) were treated as random factors. A three-way ANOVA was conducted to test for main effects and interactions. When significant effects were identified, post hoc tests were performed to determine specific differences between the groups.

For the analysis of the individual's size, a linear model was constructed to explain individual size based on genotype, followed by an ANOVA to assess the significance of the effects.

Fate of the tested animals

Post-testing, 4 dph larvae were either transferred to rearing tanks to be raised until 28 dpf for additional behavioral tests or euthanized in a bath containing embryo rearing medium enriched with 300 mg/L tricaine methane sulfonate and 600 mg/L sodium bicarbonate.

3. Results

3.1 Design of the light experimental protocol

To assess the impact of the *auts2a* gene on the response of larvae to stress induced by light, it was essential to develop an experimental protocol that would enable the reactions of the larvae to be clearly identified. This was done exclusively with individuals of the WT genotype. Different tests were conducted to establish this protocol. The variable 'Time spent in motion', an indicator of larval activity (Haigis et al., 2022), was used to assess these reactions.

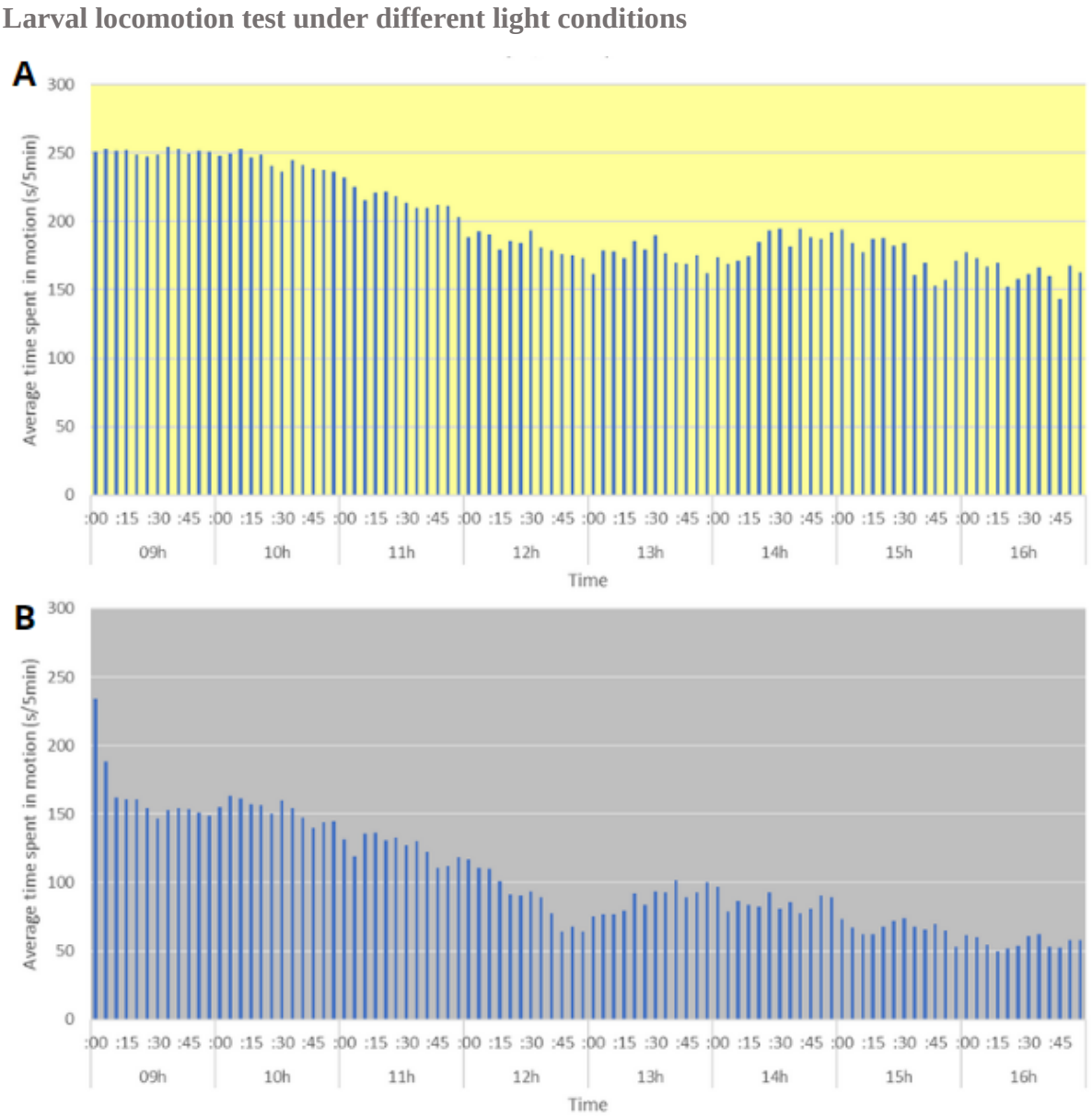


Figure 7: Evolution of the average Time spent in motion under two light conditions. A: light condition (8h, 300 lux) in Zebrabox 12 – well plates by WT medaka (*Oryzias latipes)* larvae (4 dph, n = 24), **B**: dark condition (8h, 0 lux) in Zebrabox 12 – well plates by WT medaka (*Oryzias latipes)* larvae (4 dph, n = 24).

We analyzed the activity of medaka larvae from 9:00 am to 5:00 pm under two conditions: light and darkness. These observations made it possible to distinguish the 'aversive stimulus'

triggering a stress reaction in the larvae from the ‘normal’ situation**.** Figure 7 presents the results of this locomotion tests.

In light condition (Figure 7, A), the average Time spent in motion remained stable during the first hour and a half, with larvae moving for approximately 250 seconds out of 5 minutes (83.3% of the time). After this period, the activity gradually decreased, reaching around 175 seconds of movement over 5 minutes (58.3% of the time) at the end of the test.

In darkness (Figure 7, B), the larvae's behavior exhibited significant variations. Initially, activity was high during the first 5 to 10 minutes, with larvae spending about 235 seconds in motion out of 5 minutes (78.3% of the time). Following this phase, larval activity gradually decreased and stabilized 15 min after the beginning of the test, reaching a plateau of about 2 hours, where they spent 150 seconds in motion out of 5 minutes (50% of the time). Subsequently, another decrease in activity was observed, followed by a second plateau 3 hours and 45 minutes after the beginning of the test, this time at a lower level, with only 75 seconds in motion out of 5 minutes (25% of the time). Finally, the larvae's activity continued to decline gradually until the end of the test, reaching 50 seconds in motion out of 5 minutes (16.6% of the time).

After discussion of the results (see Discussion part) we considered light as the ‘normal’ condition and darkness as an aversive stimulus.

Assessment of behavioral variability under the normal condition

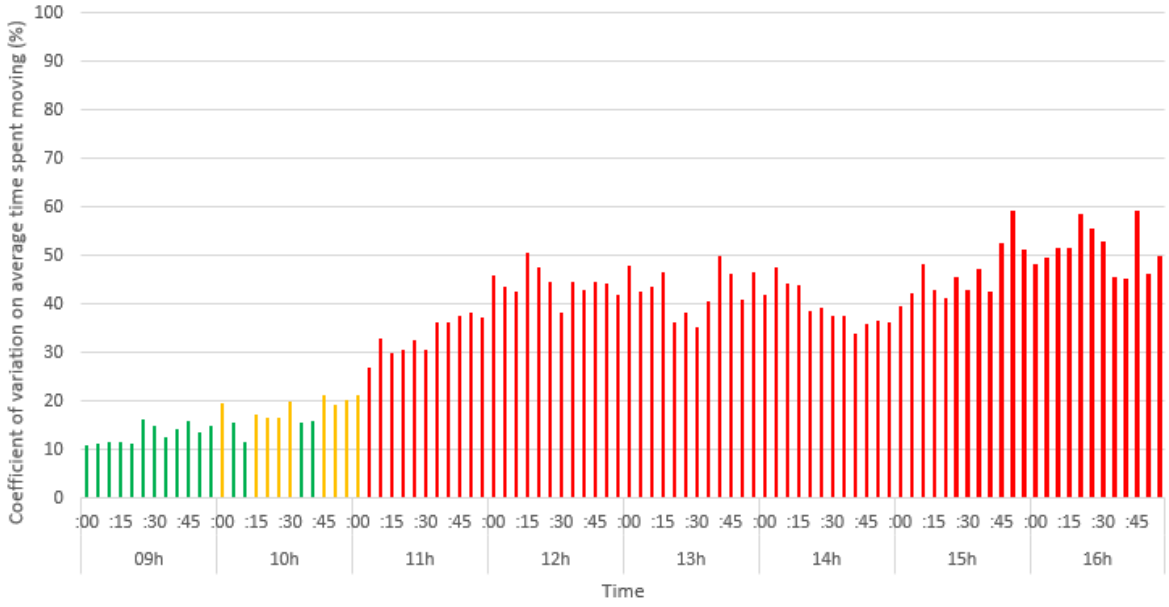


Figure 8: Evolution of the coefficient of variation on the average Time spent in motion during a day of exposure to light (8h, 300 lux) in Zebrabox 12 – well plates by WT medaka *(Oryzias latipes)* larvae (4 dph, n = 24)

We calculate the coefficients of variation (CV), a measure of the dispersion of the data in relation to the mean, of larval activity under the light condition from 9 am to 5 pm. The aim was to identify a stable phase in which the larvae exhibited homogeneous behavior. The duration of this phase is considered as the optimum duration of the test to be carried out to study behavioral differences between genotypes in response to light stimuli.

Figure 8 presents the CV values for the average Time spent in motion by larvae under normal condition. This coefficient is particularly low one hour after the beginning of the test, ranging between 10% and 20%. Beyond the first two hours following the introduction of the larvae into the Zebrabox (Viewpoint, France), the CV exceeds 20%.

Therefore, we determined that the total duration for the light exposure protocol, including the adaptation phase, should be one hour to effectively assess the behavioral response of larvae to light stimuli.

Determination of the duration of exposure to the aversive stimulus

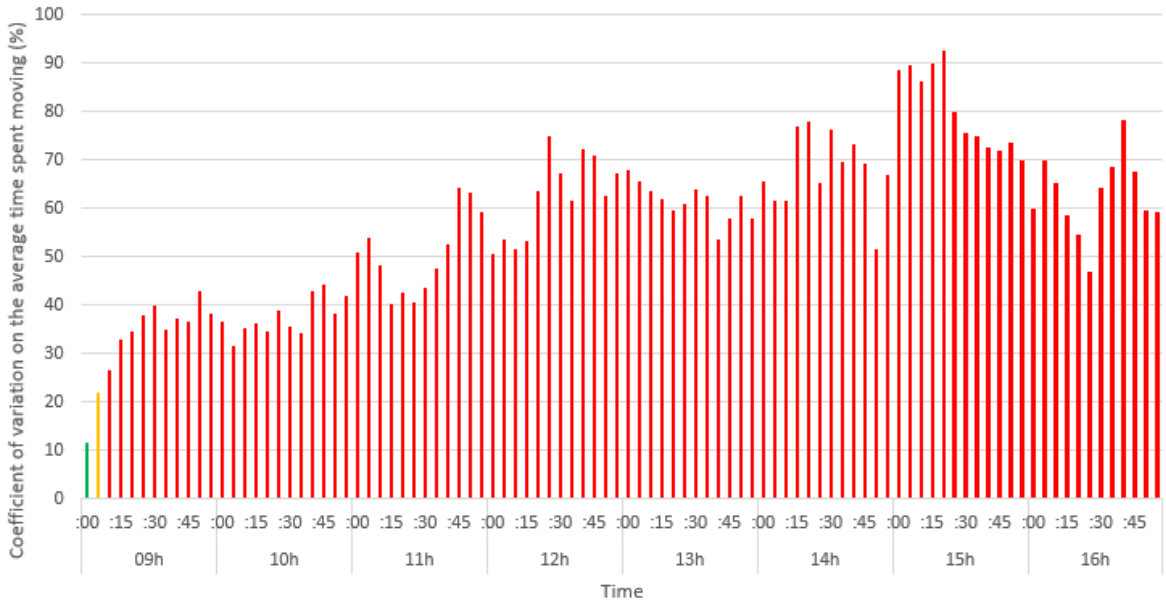


Figure 9: Evolution of the coefficient of variation on the average Time spent in motion during a day of exposure to darkness (8h, 0 lux) in Zebrabox (Viewpoint, France) 12 – well plates by WT medaka *(Oryzias latipes)* larvae (4 dph, n = 24)

We calculate the CV of larval activity under the dark condition from 9 am to 5 pm. The aim was to identify a stable phase in which the larvae exhibited homogeneous behavior. The objective here was to determine the optimal duration of the aversive stimulus to be applied in our light protocol.

Figure 9 shows that the CV for the average Time spent in motion was lowest during the first 10 minutes after the introduction of individuals into the Zebrabox (Viewpoint, France), ranging between 10% and 20%. Subsequently, this CV consistently exceeded 20%.

As the larvae exhibited consistent behavior for 10 minutes when exposed to the aversive stimulus, the duration of the dark phases within the light exposure protocol was set to 10 minutes.

Evaluation of the repeatability of homogeneous behavior under the normal condition

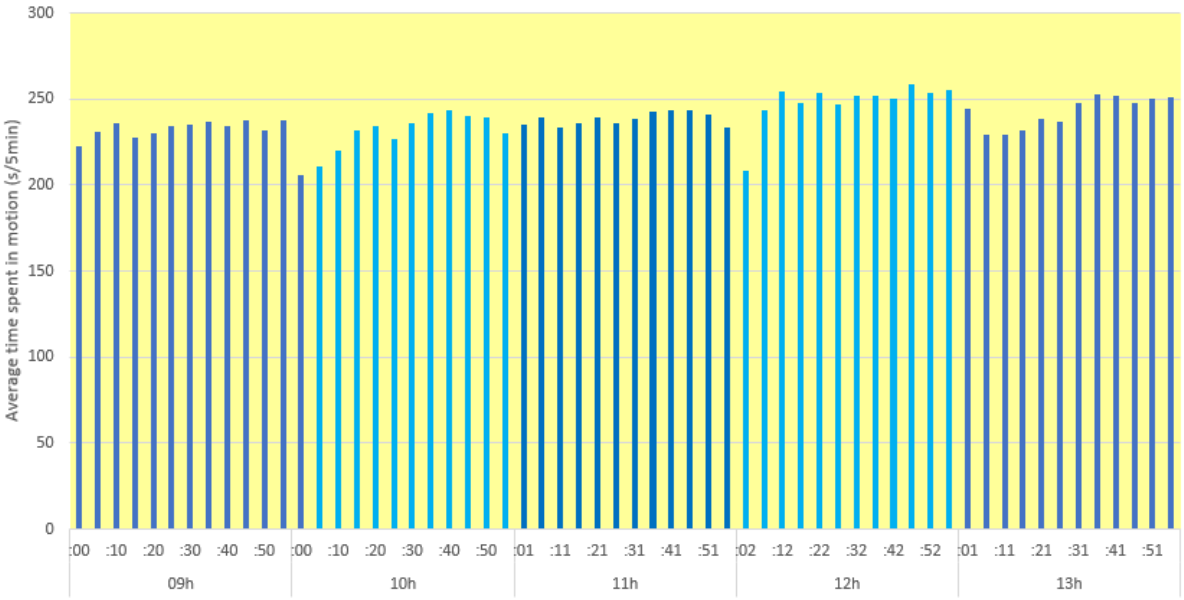


Figure 10: Average Time spent in motion during 5 successive tests of 1 hour each by WT medaka (*Oryzias latipes*) larvae (4 dph; n=12 for each test) exposed to light (300 lux) in Zebrabox (Viewpoint, France) 12-well plates.

We examined the repeatability of homogeneous larval behavior under the normal condition by observing 12 individuals from 9:00 am to 14:00 pm, with tests every hour. This allowed us to assess whether multiple tests could be conducted each day.

Figure 10 presents the results of the repeatability of the one-hour phase of homogeneous behavior under normal conditions. It is observed that, regardless of the time of introduction of the larvae into the Zebrabox, the average Time spent in motion consistently ranges between 230 seconds and 255 seconds per 5 minutes.

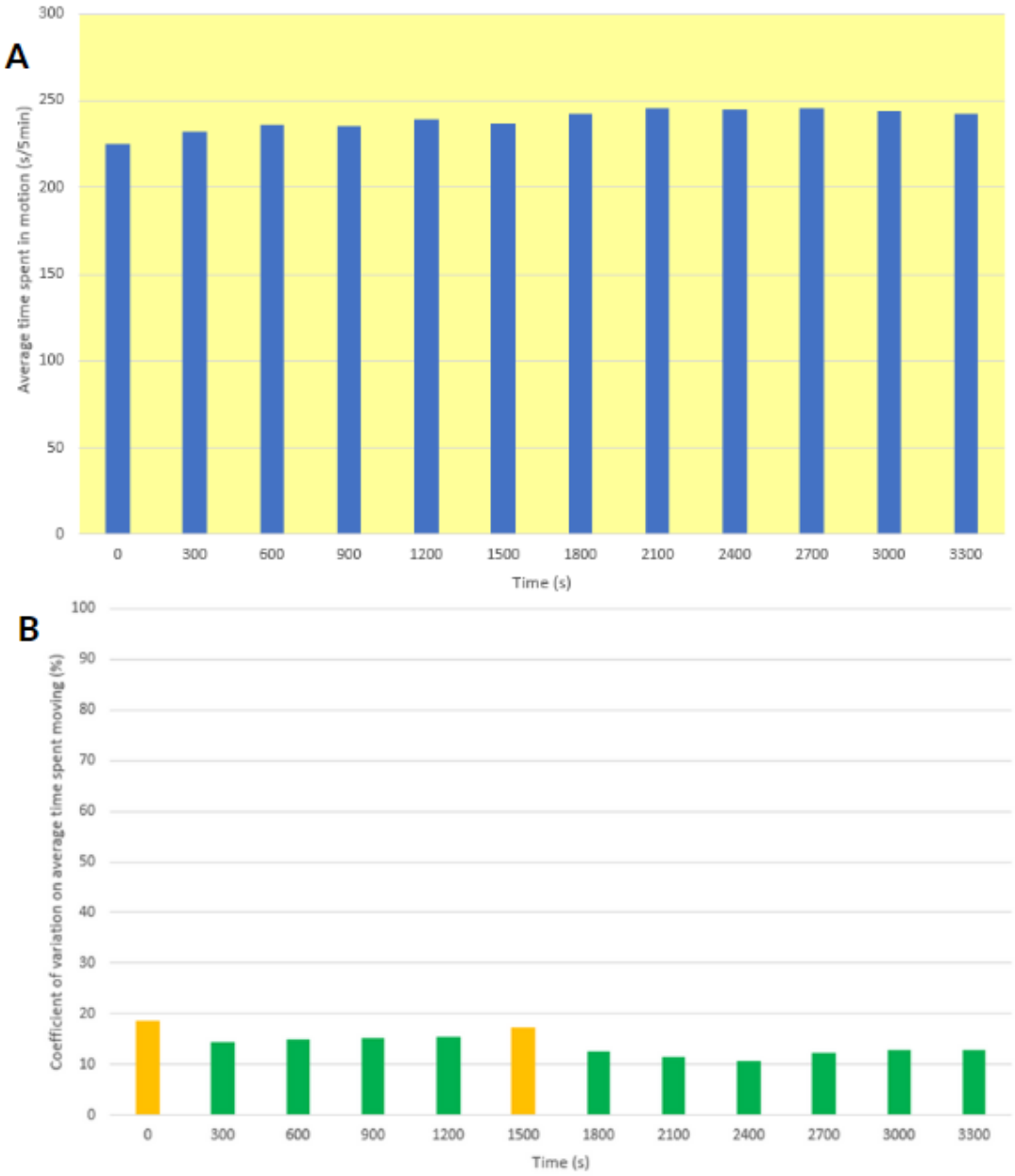


Figure 11: Average Time spent in motion and evolution of the coefficient of variation during five compiled tests of 1 hour each under light condition (300 lux). A: Average Time spent in motion during five compiled tests of 1 hour each in WT medaka (*Oryzias latipes)* larvae (4 dph; n= 60). **B**: Evolution of the coefficient of variation on the average Time spent in motion during 5 compiled tests of 1 hour each in WT medaka (*Oryzias latipes)* larvae (4 dph; n= 60), orange graph bars correspond to a coefficient of variation greater than 15%.

These observations are corroborated by Figure 11, A: the compilation of the five tests shows an average Time spent in motion ranging between 225 seconds and 245 seconds per 5 minutes throughout the full hour. Additionally, the CV consistently remains below 18% although it occasionally exceeds 15% (Figure 11, B).

Given the repeatability of this activity, multiple tests could be conducted per day without compromising the results.

Determination of the duration of adaptation to the experimental set-up

In assessing the repeatability of the behavior, we measured the time required for the individuals to regain stable activity after being introduced into the Zebrabox (Viewpoint, France). This enabled us to define the adaptation time required before subjecting the larvae to stress induced by light.

Figure 10 illustrates that 4 dph larvae take up to 20 minutes to achieve a stable average Time spent in motion. This is particularly evident in the tests conducted at 10:00 AM, 12:00 PM, and 1:00 PM.

The adaptation period for the light protocol was thus set at 20 minutes. We also chose the normal condition (light) for the adaptation phase (see Discussion part).

Final light experimental protocol

To assess potential habituation to the aversive stimulus, we decided to include repeated alternations of light and dark phases. The results of this section allowed us to establish a protocol that clearly highlights the larvae's reactions to light stimuli. This protocol is presented in Figure12.

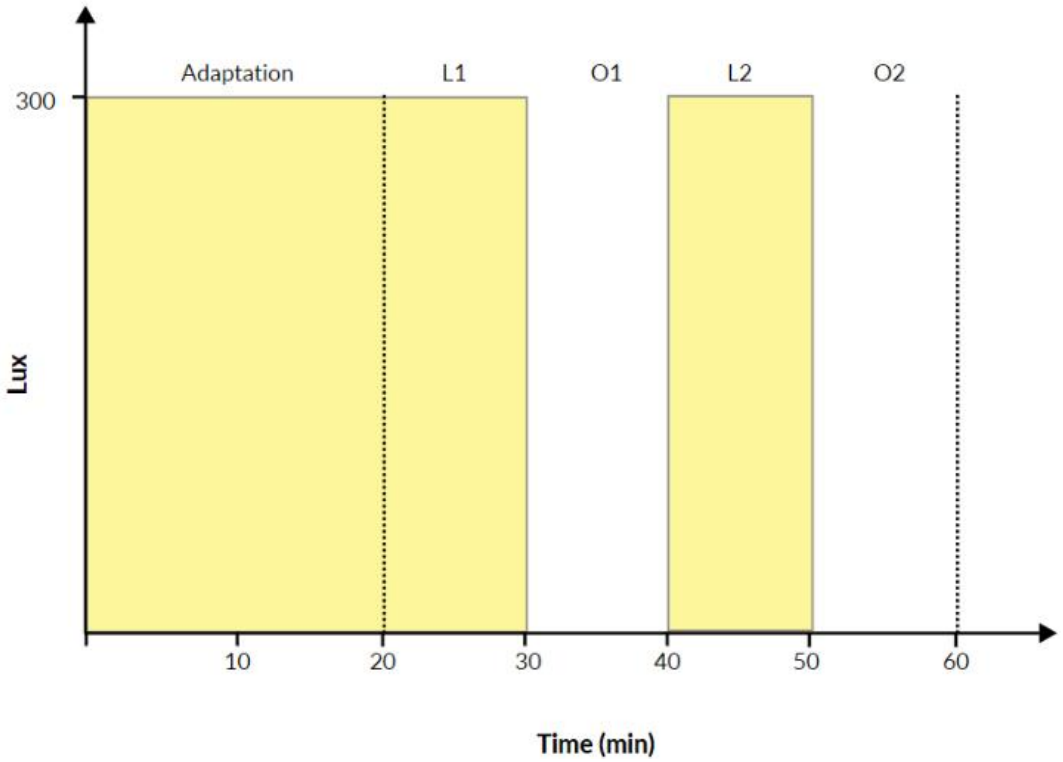


Figure 12: Light experimental protocol.

The light protocol has a total duration of one hour, starting with a 20-minute adaptation phase in the light, followed by a 40-minute tracking period divided into four phases, each lasting 10 minutes. The first phase is a light phase (L1), followed by a dark phase (O1). This alternation then repeats with another light phase (L2), and finally, a second dark phase (O2).

3.2 Evaluation of the behavioral response of larvae to light stimuli

In previous studies, 21 dpf larvae of the Δ 338k line appeared to exhibit variable sensitivities to light stimuli depending on their genotype (Trillaud 2023). We therefore continued this research to examine the presence of behavioral differences at another stage of development, 4 dph, in the same line with the light protocol established beforehand (Figure 12). Several variables were analyzed for this purpose.

To assess larval activity across the entire well, we selected the variables 'Total distance traveled' and 'Time spent in motion,' following the guidelines established in the literature (Haigis et al., 2022). We also included the count of occurrences of entry into high activity phases ('Larct'), defined by the thresholds mentioned above. These acceleration behaviors are considered strong indicators of escape behavior, light-seeking (Haigis et al. 2022), and startle responses. This 'explosive' swimming is a recognized stress reaction in fish (Maximino et al. 2010). The count of occurrences of entry into inactivity phases ('Inact') was used to evaluate the transition of individuals into a more quiescent state. To evaluate larval thigmotaxis, we examined the variable 'Time spent in the peripheral zone'.

Phase-by-phase analysis

The phase-by-phase analysis involved examining behavioral differences based on the averages of the five studied variables for each individual across all phases. This initial analysis aims to highlight the most significant differences between genotypes and identified the most discriminative variables in the overall tests

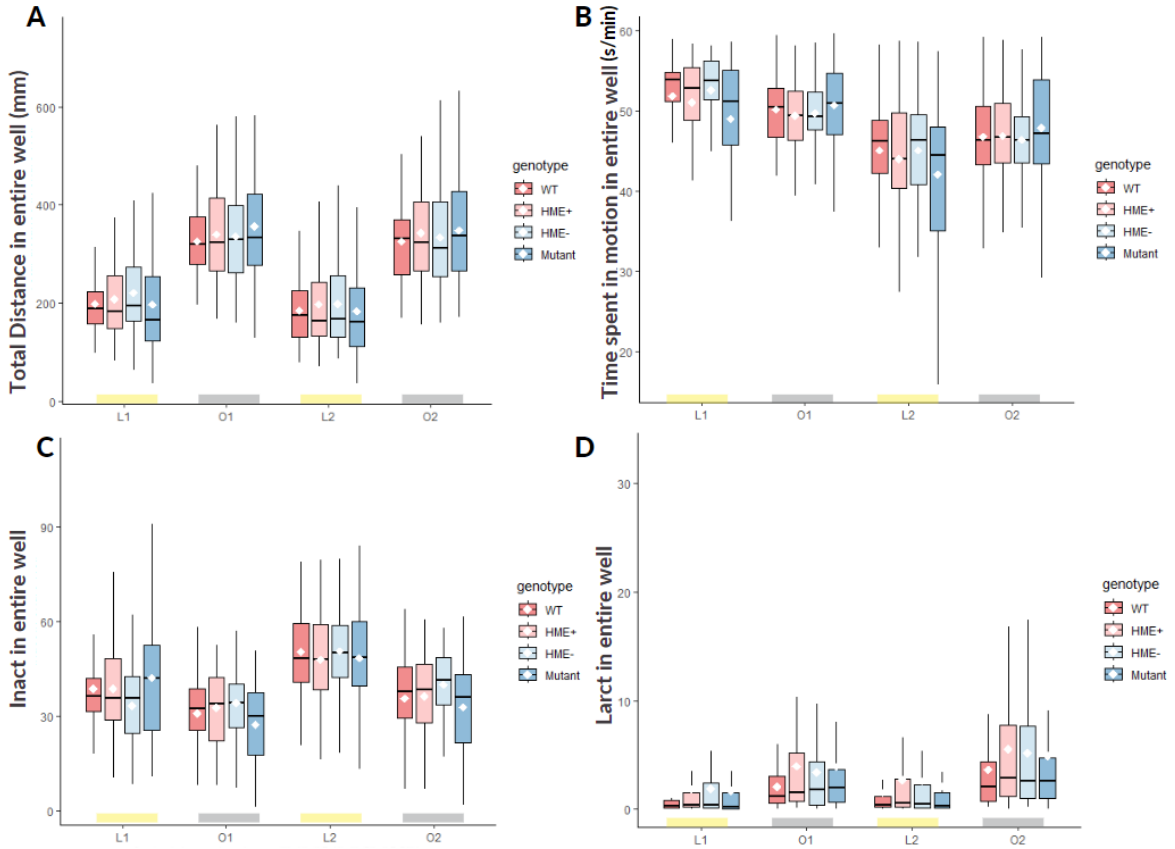


Figure 13: Boxplots of the variables studied in the entire well for the phase-by-phase study of the four genotypes (n = 260 medaka (*Oryzias latipes*) larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62). The data represent the average per individuals for each phase. **A:** Total distance (mm). **B:** Time spent in motion (s). **C:** Inactivity occurrence count. **D**: Occurrence count of high activity.

The analysis of the means for the Total distance traveled (Figure 13, A), Time spent in motion (Figure 13, B), Inact (Figure 13, C), and Larct (Figure 13, D) for each individual across all phases revealed significant interactions between phase and genotype for Total distance traveled ($\chi^2 = 22.8$, df = 9, $P < 0.001$), Time spent in motion ($\chi^2 = 34.6$, df = 9, $P < 0.0001$), and Inact ($\chi^2 = 148.4$, df = 9, $P < 0.0001$), but not for Larct ($\chi^2 = 12.7$, df = 9, $P = 0.2$). The main effect of phase is significant ($P < 0.001$) across all GLMM models. However, the main effect of genotype on the evolution of these variables was not significant ($P > 0.05$).

Across the entire well, the analysis of the means for Total distance, Time spent in motion, Inact, and Larct over all phases does not reveal any significant differences between the four genotypes (Figure 13). Therefore, a finer minute-by-minute observation of these variables during the phases of interest is required.

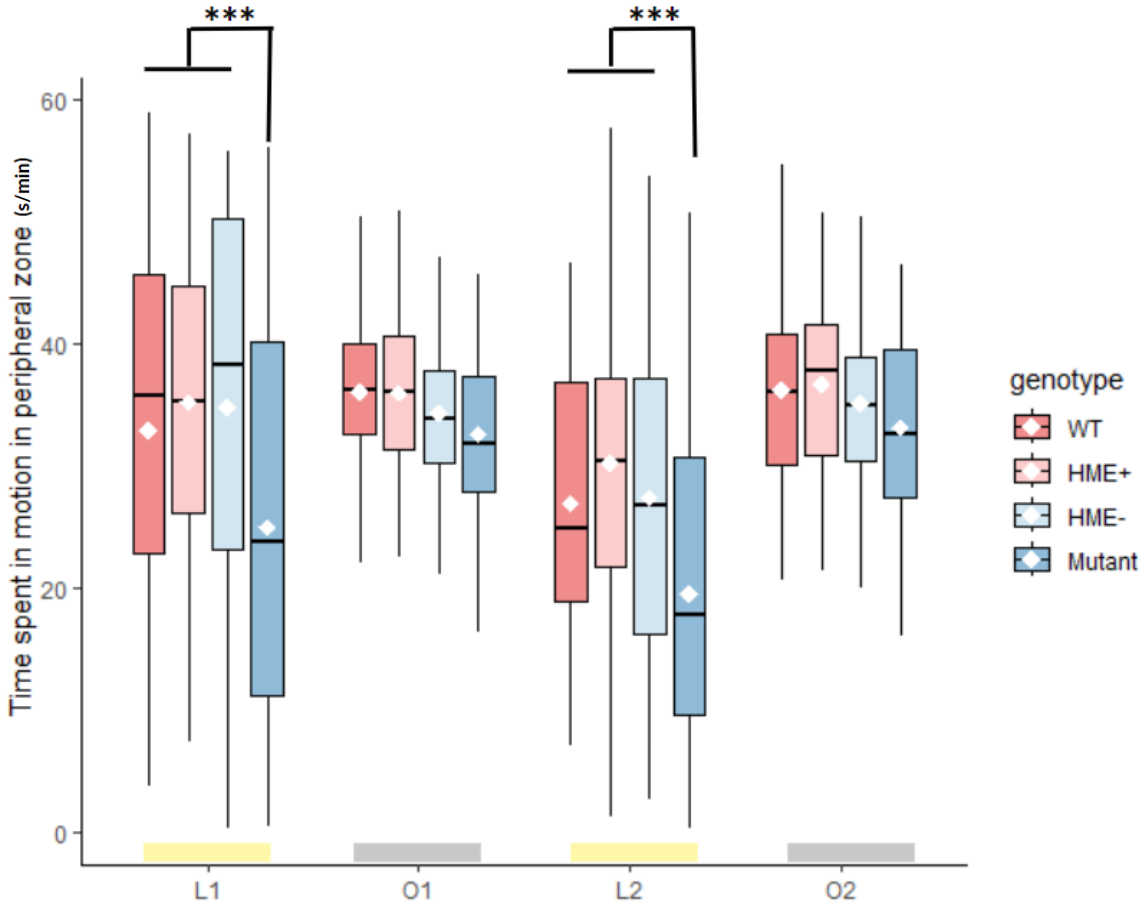


Figure 14: Evolution of the average Time spent in peripheral zone for the phase-by-phase study for the four genotypes (n = 260 medaka (*Oryzias latipes*) larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62). The data represent the average per individuals for each phase. ***P < 0.001 indicates significant differences between genotypes.

Figure 14 illustrates the evolution of the average Time spent in peripheral zone per individual in relation to genotype and phase. The selected model revealed significant interactions between phase and genotype ($\chi^2 = 129.9$, df = 9, $P < 0.001$). Both the main effects of genotype and phase are also significant ($P < 0.001$).

The analysis of the average Time spent in peripheral zone per individual in relation to genotype and phase showed significant differences between the WT, HME+, HME-, and Mutant genot ypes during the first light phase (L1) and the second (L2). The mutant genotype spends significantly less time in the peripheral zone during both L1 and L2 phases compared to the other three genotypes.

Analysis of the first dark phase

T able 1: Code used to indicate the significance of results on graphs.

	P < 0.05	P < 0.001	P < 0.0001
WT/Mutant	a	a	A
HME+/Mutant	b	b	B
HME-/Mutant	c	c	C
HME+ /WT	d	d	D
HME-/HME+	e	e	E

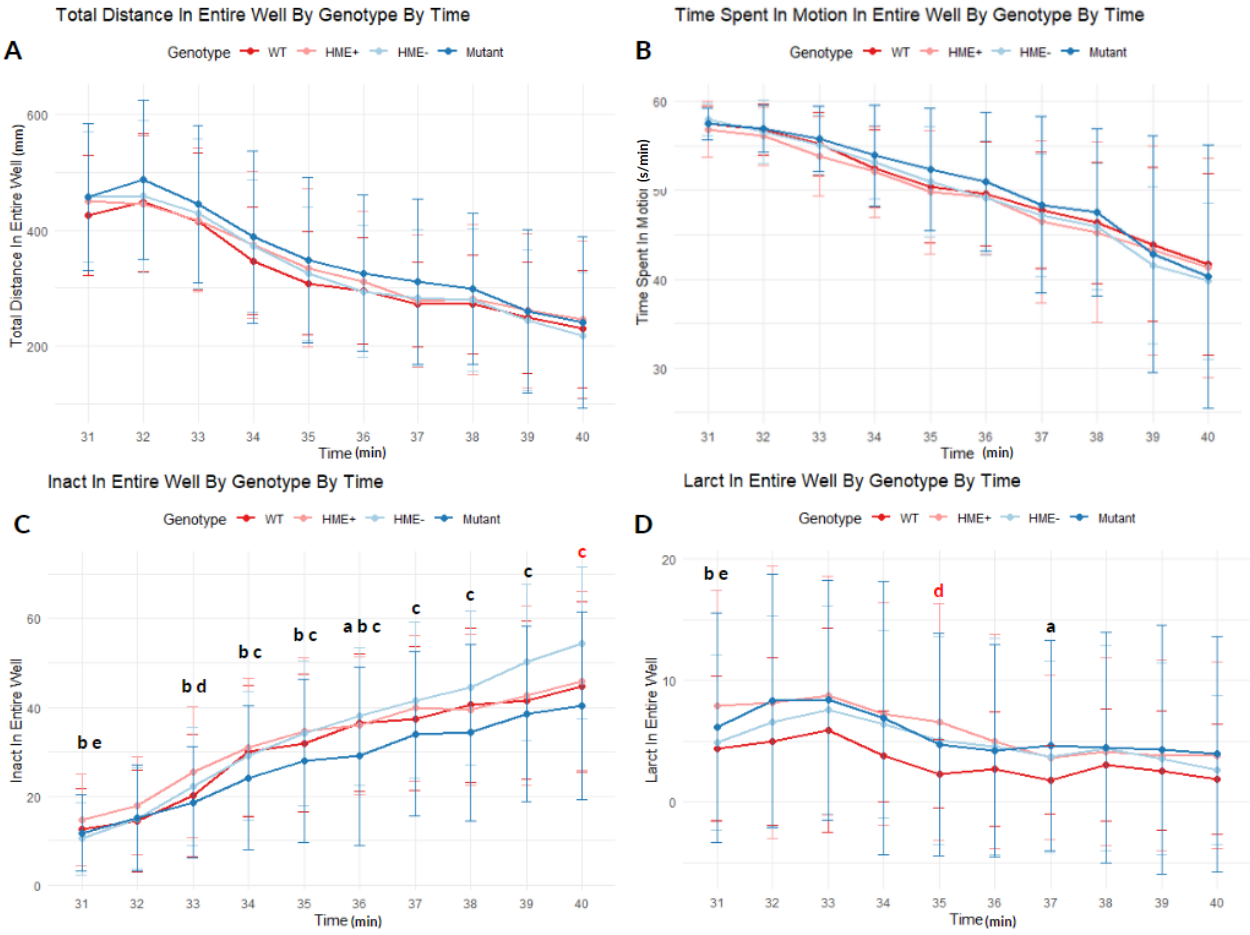


Figure 15: Variables studied in the entire well for the first phase of darkness for the four genotypes (n = 260 medaka (*Oryzias latipes*) larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62). A: Total distance (mm). **B:** Time spent in motion (s). **C**: Inactivity occurrence count. **D:** Occurrence count of high activity.

The analysis of the first dark phase aims to evaluated existing differences on a minute-by-minute basis for the five studied variables during the first dark phase (O1). This analysis provided a more detailed examination of the evolution of the five variables over time during O1 and the differences between genotypes.

The analysis of the Total distance traveled (Figure 15, A), Time spent in motion (Figure 15, B), Inact (Figure 15, C), and Larct (Figure 15, D) during O1 revealed significant interactions between genotype and time for Inact ($\chi^2 = 243.2$, df = 27, $P < 0.0001$) and Larct ($\chi^2 = 123.9$, df = 27, $P < 0.0001$). Other variables, analyzed using GLMMs with a Gamma distribution, did not show significant interactions between genotype and time. The main effect of time is significant ($P < 0.001$) across all GLMM models. However, the main effect of genotype on the evolution of Total distance and Time spent in motion is not significant ($P > 0.05$). Across the entire well, the analysis of these two variables does not show any significant differences between the genotypes.

Variable Evolution

Figure 15 highlights an initial increase of approximately 30 mm in Total distance traveled between the first and second minute for the WT and Mutant genotypes. This Total distance traveled then continuously decreases for all genotypes, reaching about 200 mm less compared to the start of the phase. The evolution of Time spent in motion is similar across the four genotypes, decreasing continuously from about 57 seconds per minute (95% of the time) to 43 seconds (72% of the time). Generally, after an initial increase, Larct decreases for all genotypes, while Inact increases. Furthermore, these two variables reveal significant differences between genotypes.

Differences Between Genotypes

Inact, highlights differences between genotypes (Table 1, Figure 15, C). The first minute of darkness shows a significant difference between the HME- and Mutant genotypes compared to the HME+ genotype, which enters inactivity significantly more frequently. At t = 33 minutes, the HME+ genotype also enters inactivity significantly more often than the WT and Mutant genotypes. After t = 33 minutes, the Mutant genotype enters inactivity significantly less frequently than the HME- and HME+ genotypes initially (at t = 34 minutes, t = 35 minutes) and then compared to all three other genotypes together (at t = 36 minutes). After t = 36 minutes and until the end of O1 (t = 40 minutes), a significant difference remains between the HME- genotype and the Mutant genotype, which continues to enter inactivity significantly less frequently.

Larct also highlights significant differences between genotypes (Table 1, Figure 15, D). The HME+ genotype enters high activity significantly more often than the HME- genotype in the first minute. At t = 35 minutes, the HME+ genotype also enters high activity significantly more frequently than the WT genotype. Finally, at t = 37 minutes, the Mutant genotype enters high activity significantly more often than the wild-type genotype.

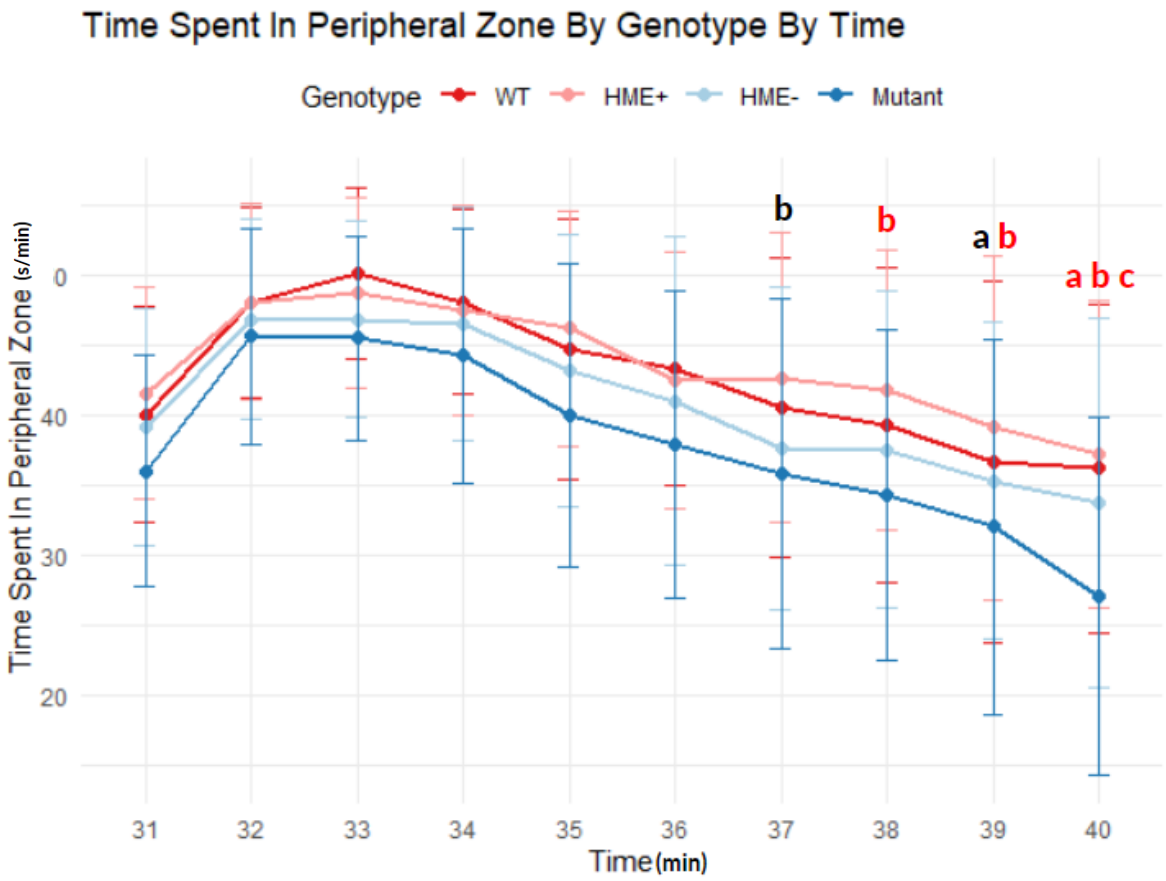


Figure 16: Evolution of the average Time spent in peripheral zone for the first phase of darkness for the four genotypes (n = 260 medaka (*Oryzias latipes*) larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62).

Figure 16 shows the evolution of Time spent in peripheral zone in relation to genotype and time during O1. The chosen model revealed significant interactions between phase and genotype (χ^2 = 47.8, df = 27, P < 0.01). The main effect of time is also significant (P < 0.001), but the main effect of genotype is not (P = 0.08).

Evolution of Time spent in peripheral zone

The analysis of Time spent in peripheral zone highlights an increase of approximately 10 seconds (16,7%) during the first two minutes of O1 for all genotypes. Values then decrease for all four genotypes, but with significant differences.

Differences between genotypes

Indeed, larvae of the Mutant genotype stand out by spending significantly less time in the peripheral zone compared to larvae of the HME+ genotype (t = 37 min, t = 38 min; t = 39 min, t = 40 min), WT genotype (t = 39 min, t = 40 min), and finally, the HME- genotype (t = 40 min) (Table 1, Figure 16).

Summary of results for the analysis of O1:

Thus, the minute-by-minute analysis of the five variables during O1 highlighted major trends across the four genotypes: beyond the first minute of exposure, Total distance traveled during O1 decreases over time. The same trend is observed for Time spent in motion and Time spent in peripheral zone. Inact, however, progressively increases over time. Significant differences not observable from the phase-by-phase average analysis were also revealed, notably for the two counting variables and Time spent in peripheral zone. Besides the specific differences

highlighted by Larct, the variables Inact and Time spent in peripheral zone demonstrate that the Mutant genotype differs from the others with a lower entry into inactivity and less Time spent in peripheral zone.

Analysis of the second dark phase

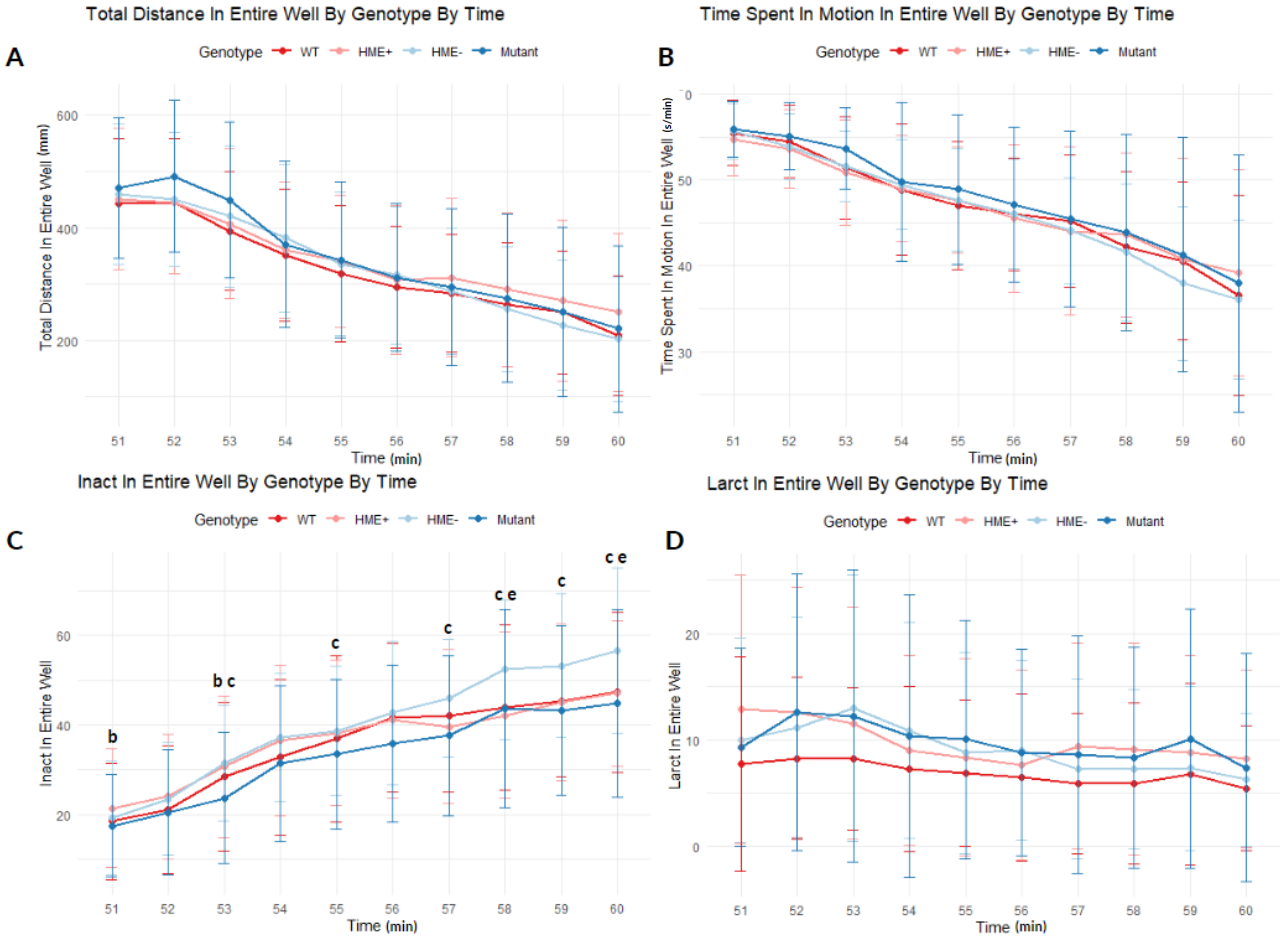


Figure 17: Variables studied in the entire well for the second phase of darkness for the four genotypes (n = 260 medaka (*Oryzias latipes*) larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62). A: Total distance (mm). **B:** Time spent in motion (s). **C**: Inactivity occurrence count. **D:** Occurrence count of high activity.

To assess the potential persistence of the trends and differences observed in O1, we analyzed the evolution of the variables on the second dark phase (O2).

Figure 17 presents the evolution of Total distance (Figure 17, A), Time spent in motion (Figure 17, B), Inact (Figure 17, C), and Larct (Figure 17, D) during O2. Significant interactions between genotype and time were observed for Total distance ($\chi^2 = 67.1$, df = 27, $P < 0.0001$), Inact ($\chi^2 = 173.3$, df = 27, $P < 0.0001$), and Larct ($\chi^2 = 136.5$, df = 27, $P < 0.0001$). The model used to explain Time spent in motion did not show a significant interaction ($P = 0.7$). The main effect of time is significant ($P < 0.001$) across all GLMM models. However, the main effect of genotype on the evolution of the four variables is not ($P > 0.05$).

Evolution of variables

The variables generally exhibit similar trends to those observed in O1. However, the increase in activity noted in the first few minutes with Total distance traveled in O1 is no longer visible in WT genotype larvae. Only Mutant genotype larvae show an increase of about 20 mm in Total distance traveled during the first minute of O2.

Comparison of values with O1

Comparing values between O1 and O2 suggests a lower Time spent in motion for all genotypes during O2. Additionally, the counting variables (Inact and Larct) are also higher during this phase.

Differences between genotypes

Like the findings in O1, Inact in O2 highlights significant differences between genotypes (Table 1, Figure 17, C). The first minute of O2 shows a significant difference between Mutant and HME+ genotypes, with HME+ entering inactivity significantly more often. At t = 53 minutes, heterozygous genotypes enter inactivity significantly more than the Mutant genotype. Similarly, the end of O2 is marked by significantly higher inactivity in HME- individuals compared to the Mutant genotype. A persistent difference between heterozygous genotypes is also observed: at t = 58 min and t = 60 min, HME- larvae enter inactivity significantly more than HME+ larvae. However, this difference is reversed compared to what is observed in the first minute of O1. In O2, Larct (Figure 17, D) no longer shows significant differences between genotypes.

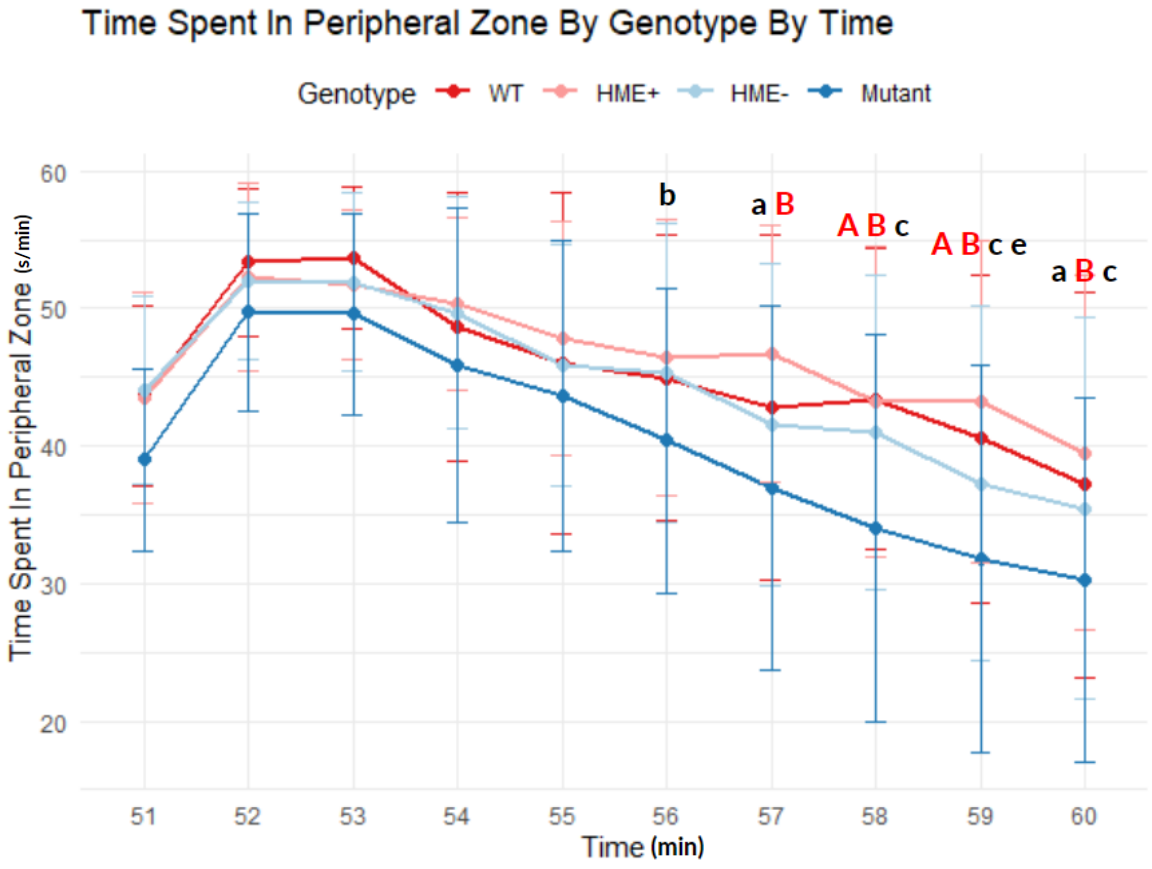


Figure 18: Evolution of the average Time spent in peripheral zone for the second phase of darkness for the four genotypes (n = 260 medaka (*Oryzias latipes*) larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62).

Figure 18 illustrates the evolution of Time spent in peripheral zone as a function of genotype and time during O2. The chosen model revealed significant interactions between phase and genotype ($\chi^2 = 72.9$, df = 27, $P < 0.01$). The main effect of time is also significant ($P < 0.001$), but the main effect of genotype is not ($P = 0.1$).

Evolution of Time spent in peripheral zone

Time spent in peripheral zone during O2 follows a similar pattern to that observed in O1: an initial increase of about 10 seconds followed by a decrease, with differences among genotypes.

Comparison of values with O1

The comparison of Time spent in peripheral zone values between O1 and O2 suggests that all genotypes spend more time in the peripheral zone during phase O2.

Differences between genotypes

Consistent with O1, Time spent in peripheral zone reveals that Mutant genotype larvae stand out by spending significantly less time in the peripheral zone compared to HME+ larvae (t = 56 min, t = 57 min; t = 58 min, t = 59 min, t = 60 min), WT larvae (t = 57 min; t = 58 min, t = 59 min, t = 60 min), and HME- larvae (t = 58 min, t = 59 min, t = 60 min). Additionally, at t = 59 min, HME+ larvae spend significantly more time in the peripheral zone compared to HME- larvae, a difference not observed during O1 (Table 1, Figure 18).

Summary of results in O2

The minute-by-minute analysis of the five variables during O2 partially mirrored the results from O1. However, the analysis of the end of O2 highlights more pronounced differences compared to those observed in O1. Moreover, new differences among heterozygous genotypes emerged concerning Time spent in peripheral zone, which is significantly lower in HME- larvae, and Inact, which is significantly higher for these same individuals.

Analysis of individual's size

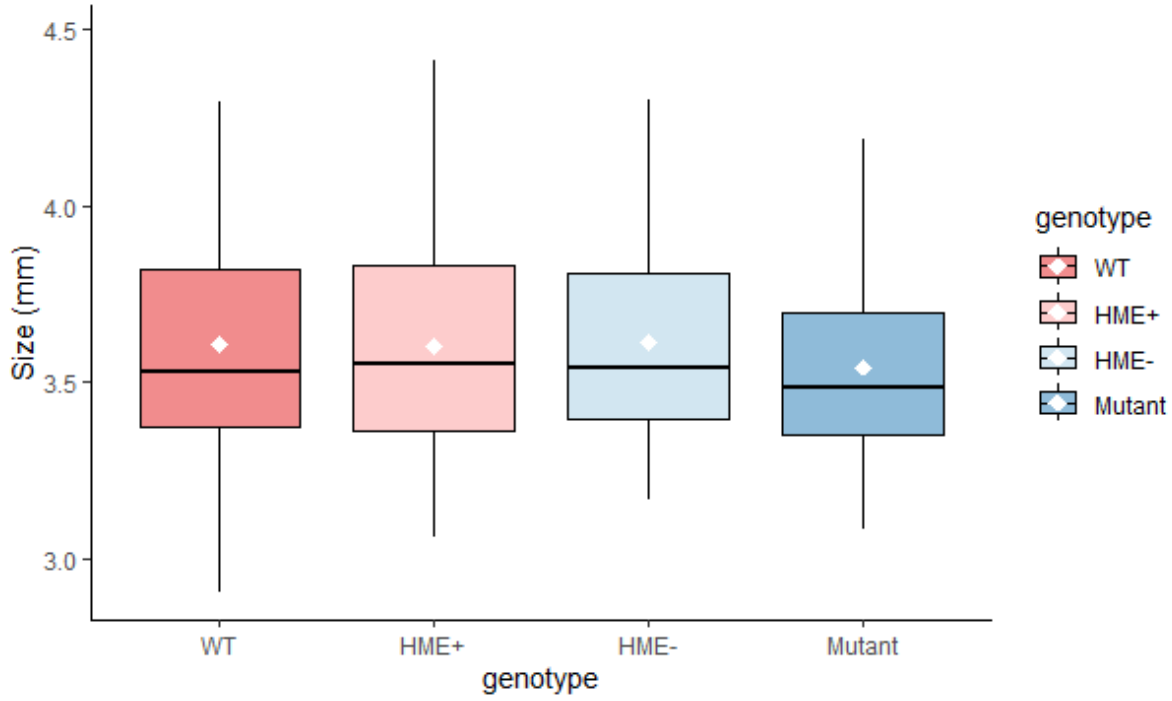


Figure 19: Size of 4dph medaka (*Oryzias latipes)* larvae evaluated by genotypes (n = 260 *medaka* larvae: WT: n = 66, HME+: n = 70, HME-: n = 62, Mutant: n = 62).

Preliminary results of the project showed that the absence of maternal expression of the auts2a gene affected the size of the individuals (Clément 2023). To ensure that the differences observed in our study were not related to morphological differences, we compared the size of individuals across genotypes.

Figure 19 presents the sizes of the tested individuals by genotype. The model revealed no significant size differences between the different genotypes (P = 0.462).

4. Discussion

4.1 Design of the light experimental protocol

Larval locomotion test under different light conditions

The results from the locomotion test revealed a distinct behavioral response of medaka larvae to light stimuli. Under light condition, the larvae show stable activity during the first hour and a half, followed by a gradual decrease. In contrast, in darkness, the larvae exhibit high initial activity that then decreases to a plateau before declining further. This response pattern is consistent with observations in other fish species, such as the zebrafish, where a phase of activity is followed by a decrease and stabilization of activity in response to aversive stimuli like darkness (MacPhail et al. 2009; Kristofco et al. 2016). The results obtained here confirm that darkness constitutes an aversive stimulus for medaka larvae, eliciting a stress response characterized by initially high activity followed by a progressive strong decrease.

Assessment of behavioral variability under the normal condition

Analyses revealed that the larvae exhibit consistent behavior during the first hours after introduction into the Zebrabox, with relatively low coefficients of variation in the Time spent in motion (10-20%). This stable phase is crucial for defining the total test duration to study behavioral differences in response to light stimuli. In comparison with other studies on medaka larvae that use longer test durations (Le Bihanic et al., 2014; Chiffre et al., 2016), our results indicate that the stable phase can be maintained for approximately one hour, suggesting a different approach for defining experimental light protocols.

Determination of the duration of exposure to the aversive stimulus

Our results show that the first 10 minutes of exposure to darkness induce consistent activity among the larvae, allowing for reliable assessment of behavior in response to this aversive stimulus. This duration is shorter than the darkness exposure periods typically used in other protocols for medaka larvae (Le Bihanic et al., 2014; Chiffre et al., 2016), but comparable to studies on zebrafish where shorter exposure durations are also employed (Vignet et al., 2013). Utilizing a 10-minute period in our protocol allows us to focus the analysis on the initial behavioral response without introducing potential variability due to prolonged acclimation.

Evaluation of the repeatability of homogeneous behavior under the normal condition

The repeatability of the homogeneous behavior phase was confirmed with consistent Time spent in motion across multiple tests conducted successively in the morning. The results indicate that the stable phase can be reliably reproduced during the day, which means that the test can be conducted several times a day. This observation aligns with common practices of conducting multiple tests per day in Zebrabox protocols (Viewpoint, France) (Chiffre et al. 2016; Le Bihanic et al. 2014). This observation suggests that the time of day does not significantly affect the homogeneous behavior phase and allows for multiple experiments to be conducted within the same session.

Determination of the duration of adaptation to the experimental set-up

Our study results show that medaka larvae require up to 20 minutes to reach a stable level of activity after their introduction into the Zebrabox (Viewpoint, France). This observation is crucial for ensuring accurate and reproducible measurements of behavior. Compared to existing literature, our results suggest that the recommended acclimation period in some previous studies may be considered excessive for the experimental conditions we have implemented. This duration is consistent with what is observed in the literature. Indeed, this duration was also chosen by Chiffre et al. (2016), in contrast to Le Bihanic et al. (2014), who recommended a one-hour acclimation period. This reduction in the acclimation phase represents an optimal compromise between precision and operational efficiency in the context of our study.

Choice to conduct the adaptation phase in light condition

We opted for a acclimation phase in light, unlike other protocols involving medaka, which use darkness for this phase (Le Bihanic et al. 2014; Chiffre et al. 2016; Dasmahapatra, Carty, Khan 2017). This choice aims to prevent larvae from becoming habituated to the dark stimulus, which is considered aversive in this study, potentially affecting the results related to their response to darkness. It is also noted that some studies on zebrafish use a light acclimation phase, which may enhance larval activity (MacPhail et al. 2009; Schnörr et al. 2012; Vignet et al. 2013). By exposing the larvae to light during the acclimation phase, we aim to maintain a clearer behavioral response to changes in light conditions, thereby avoiding potential habituation to aversive stimuli that could compromise the integrity of the data obtained.

Limitations in the development of the experimental protocol

The development of the experimental protocol does have some limitations. Specifically, the tests were conducted between 9:00 AM and 5:00 PM, and we differentiated between the aversive condition and the so-called "normal" condition based on this time frame. However, it is possible that these results are only valid within this time window, as larval behavior may be influenced by circadian variations, which have been demonstrated in medaka (Hata et al. 2024).

Furthermore, only 24 larvae were used for most of the tests designed to develop the experimental protocol. This small sample size limits the study's power, potentially making the results less representative and generalizable. A larger sample size would be necessary to enhance the reliability of the conclusions and better capture biological variability.

4.2 Evaluation of the behavioral response of larvae to light stimuli

Evolution of variables over time during darkness

Analysis of the evolution of variables during the two phases of darkness revealed general trends observable across all genotypes. In O1, after an initial increase, the Total distance traveled, Larct, and Time spent in the peripheral zone decrease over time. Some genotypes thus exhibit an initial increase in activity and all four genotypes exhibit an initial increase in thigmotaxis, indicating a significant rise in anxiety-like behaviors. This supports the hypothesis that the aversive stimulus induces an intense stress response in all genotypes and explains why phase-by-phase analysis could not reveal significant differences during the dark phase. The observed reactions are typical of an alarm phase characterized by a neuroendocrine cascade allowing the fish to express, among other things, escape behaviors (Schreck, Tort 2016). This is an innate anti-predator defensive behavior (Hall, Suboski 1995). This response can also be referred to as a "startle reaction" and has been observed in zebrafish larvae when transitioning from light to darkness (Emran, Rihel, Dowling 2008; Colwill, Creton 2011b).

This initial phase is followed by a decline in the Total distance traveled, Time spent in motion, Time spent in peripheral zone, and an increase of Inact over time for all genotypes. Additionally, although slight and with high standard deviations, a decrease in Larct is also observed. This phenomenon can be interpreted as the implementation of various protective strategies. After the initial alarm phase, the sustained darkness, perceived as a stressful environment, may trigger a defensive response in which the larvae reduce their movement to minimize predation risks, thus adopting a camouflage strategy (Schreck, Tort 2016). This decrease in activity could also be interpreted as a form of resignation, where the larvae cease to act after having "learned" that their actions do not influence the aversive stimulus. Furthermore, darkness might prompt them to conserve their energy (Schreck, Tort 2016).

Differences between genotypes

The results of this study on 4 dph medaka larvae provide insights into the role of the *auts2a* gene in the regulation of behavior, particularly regarding Time spent in the peripheral zone and Inact. By comparing four genotypes (WT, HME+, HME-, and Mutant), the study highlights significant differences that enhance our understanding of the behavioral determinism associated with *auts2a* in medaka.

Thigmotaxis and anxiety

The creation of a peripheral zone in the Zebralab software and the study of the time spent in this zone allowed for the assessment of the thigmotactic behavior of the larvae. The results indicate that mutant larvae spend less time in the periphery, both during the light phases (L1 and L2) and at the end of the dark phases (O1 and O2). This suggests a reduction in thigmotaxis, and by extension, a possible decrease in anxiety in mutant larvae. These findings are consistent with previous studies demonstrating reduced thigmotaxis in mutant genotype larvae at 21 dph within the project framework (Trillaud 2023). However, this is contrary to studies conducted in mice (*Mus musculus*). Open field tests have shown that ASD-affected mice spend less time in the center of the arena compared to the wild-type one, indicating increased anxiety-related behavior in ASD-affected mice (Silverman et al. 2011; Simmons et al. 2021; Tamada et al. 2010).

Additionally, a difference is observed between HME- and HME+ genotypes at the end of the O2 phase, with HME- larvae spending less time in the periphery. This highlights the significance of maternal expression of the *auts2a* gene in the development of anxiety-related behaviors in medaka larvae. These findings are consistent with conclusions drawn from studies on medaka diving behavior within the project, where larvae lacking maternal contribution of the gene exhibited reduced anxiety-related behaviors (Clément 2023). However, the lack of a significant distinction between HME- and WT genotypes makes it challenging to draw definitive conclusions about the impact of maternal contribution of the *auts2a* gene on thigmotaxis. This point requires further detailed analysis. Moreover, the difference between heterozygous genotypes is observable only during one minute of the second dark phase, suggesting that additional analyses are needed to confirm this trend.

Finally, while thigmotaxis has been demonstrated in zebrafish larvae (Colwill, Creton 2011a; Schnörr et al. 2012), it is important to note that one study showed this behavior only appears in medaka at 120 dph, suggesting that this behavior may develop later in the larvae's life cycle (Lucon-Xiccato et al. 2022). Despite this temporal difference, the presence of thigmotaxis in our study with younger larvae confirms an anxious response to aversive stimuli, indicating that even at an early stage, medaka can exhibit stress responses like those observed at more advanced stages.

Implemented protective strategies and maternal contribution

During the O1 phase, mutant larvae exhibit significantly reduced inactivity compared to other genotypes. This suggests that the protective mechanisms observed in other genotypes such as reducing movement to minimize predation risk or a form of resignation, are impaired when the *auts2a* gene is homozygously mutated. Mutants therefore appear less inclined to adopt typical protective behaviors, indicating weaker protective mechanisms in larvae of this genotype.

In contrast, HME- larvae display a distinct inactivity profile. In the O1 phase, they are particularly more inactive than mutants, and this trend intensifies in the O2 phase, where HME- larvae show significantly higher inactivity than both mutant larvae and HME+ larvae. This difference underscores the importance of maternal contribution of the *auts2a* gene. The results suggest that this maternal contribution promotes spatial protective strategies, such as thigmotaxis. In contrast, the mere absence of this maternal contribution (as seen in HME-) seems to favor different protective strategies, such as reduced movement to minimize predation risk, resignation, or energy conservation in response to a perceived stressful environment.

In conclusion, the absence of maternal contribution alone, observed in HME- larvae, might favor distinct protective strategies, such as increased inactivity, compared to the spatial protective strategy (thigmotaxis) observed in larvae with maternal contribution of the *auts2a* gene. Mutant larvae, lacking both maternal and paternal contributions of the gene, exhibit weaker expression of both types of strategies. It is not novel to find that alteration in a maternal factor leads to atypical spatial preferences (Moravec et al. 2016) and this interpretation is consistent with previous results from the project. Indeed, it has been shown that individuals lacking maternal contribution of the gene display reduced geotaxis and poorer spatial recognition of their environment (Clément 2023). These observations suggest that the absence of maternal contribution of *auts2a* not only affects spatial recognition but also spatial behavioral regulation mechanisms in response to aversive stimuli. However, no studies have shown that the absence of maternal contribution alone is responsible for increased inactivity. This complex adaptive process therefore requires further exploration to understand the underlying mechanisms responsible for this observation within the project framework.

Impact of repeated exposure to aversive stimuli on behavioral responses

During the O2 phase, where the aversive stimulus was repeated, all genotypes exhibited a more pronounced stress response, characterized by a general increase of Inact, Larct, and of the Time spent in the peripheral zone compared to the O1 phase. This observation suggests that the repetition of the stimulus does not lead to habituation but rather reinforces resignation strategies, escape behaviors, and thigmotaxis. The larvae appear to have "learned" that darkness is stressful, which amplifies their response during the second exposure.

However, the increase in thigmotaxis during O2 was less pronounced in mutant larvae, highlighting even more significant differences between them and the other genotypes. This reduced increase in thigmotaxis among mutants confirms that their anxiety levels are lower than those of other genotypes, even in response to the repeated aversive stimulus.

Additionally, the second phase of darkness also revealed behavioral differences between the heterozygous genotypes. The HME- larvae showed higher inactivity and less pronounced thigmotaxis than the HME+ larvae. This confirms that the repetition of the aversive stimulus further accentuates the choice of protection strategies, making the differences between genotypes more apparent.

Thus, the repetition of the dark phase in the experimental protocol allowed for clearer behavioral differences between the genotypes to emerge. The value of this repetition lies in its ability to amplify and better characterize stress response strategies, providing valuable insights into the role of the *auts2a* gene in regulating behaviors in response to an aversive stimulus. These results suggest that the repetition of the aversive stimulus is a useful approach for deepening the understanding of underlying behavioral mechanisms, particularly those related to resignation, thigmotaxis, and other forms of stress responses. It would be interesting to repeat the dark phase further to observe how these strategy choices evolve over time and determine whether a potential habituation phenomenon might occur, thereby offering an even more nuanced understanding of behavioral dynamics.

Size of Individuals

Unlike the phenotypes observed in Humans (Beunders et al. 2013), murine models (Gao et al. 2014) or in adult medaka (Clément 2023), medaka larvae lacking maternal contribution of the *auts2a* gene at the 4dph stage are not smaller in size. These findings suggest that this morphological difference appears later in development. This observation is crucial for the interpretation of behavioral results, as it implies that the variations observed in stress responses cannot be attributed to morphological differences between experimental groups.

Limitations of assessing larval behavioral responses to light stimuli

While this study provides valuable insights into the behavioral responses of medaka larvae to light stimuli, several limitations must be considered when interpreting these findings.

Firstly, although larvae were arbitrarily selected from petri dishes to the experimental setup, the manual selection process using a pipette introduces variability. The fact that this selection was made by a human could have unintentionally introduced bias, potentially compromising the perfect randomization of the individual selection. This potential bias may have influenced the observed results by introducing uncontrolled differences between experimental groups.

Secondly, despite the experimental setup being placed in an isolated room, the lack of soundproofing walls questions about the possible impact of extraneous noise on the larvae's

behavioral responses. The possibility that auditory stimuli may have influenced the results cannot be ignored.

Thirdly, the decision to focus on a very specific developmental stage, 4 dph, raises certain concerns. Notably, differences in hatching times were observed among the genotypes studied, with mutant medaka eggs showing a delayed hatching compared to other genotypes. While selecting this specific stage is crucial for ensuring homogeneity in the developmental stage of the individuals, it may have introduced a selection pressure that could potentially influence the observed behavioral differences between genotypes. In other words, this stage choice may have favored certain individuals while excluding others, potentially explaining, at least in part, the behavioral variations between genotype groups.

Moreover, the experimental approach primarily targeted early behavioral responses, focusing on the initial minutes of stimulus exposure. While this phase is critical for understanding initial reactions, it does not allow for the assessment of longer-term behavioral evolution. Extended studies would be necessary to determine whether larvae develop habituation or other forms of adaptation to stimuli over time.

In addition, the analysis relied on specific behavioral measures, such as Total distance traveled, Time spent in motion, and thigmotaxis, while excluding other potential indicators of stress or anxiety. Integrating physiological measures, such as cortisol levels or other stress biomarkers, could enhance the understanding of the underlying mechanisms driving the observed behaviors.

It is also important to note that the responses observed in this study are limited to light stimuli. Evaluating the larvae's reactions to other types of stimuli, such as auditory or tactile stimuli, would provide a more comprehensive view of their behavior and stress response mechanisms.

The experimental conditions must also be considered when interpreting the results. The larvae were isolated in an experimental setup and placed in a novel environment. This inherently stressful situation may have influenced their behavior, even in the condition considered "normal" (light). These potential stress factors raise questions about the validity of this condition as a baseline for comparison and the generalizability of the observed results.

Finally, it would be interesting to evaluate the behavioral responses at a different developmental stage to compare the differences between the 4 dph stage and more advanced stages. The behaviors observed at an early stage may evolve over time, particularly concerning thigmotaxis and stress responses, which may manifest differently as the larvae develop. This perspective would deepen our understanding of the maturation of behavioral mechanisms and the evolutionary role of the *auts2a* gene throughout development.

Conclusion

This study has expended our understanding of the behavioral responses of medaka larvae (*Oryzias latipes*) to light stimuli, highlighting the significant role of the *auts2a* gene in regulating anxiety-related behaviors. The findings demonstrate that darkness induces a behavioral response characteristic of exposure to an aversive stimulus, marked by an initial phase of increased activity followed by a gradual decline. Additionally, the observed differences between genotypes, particularly in terms of thigmotaxis and inactivity, highlights the crucial role of maternal contribution of the *auts2a* gene in the larvae's behavioral adaptation.

One of the major accomplishments of this research is the establishment of a robust and reproducible experimental protocol within the laboratory. The developed protocol effectively characterized the behavioral responses of larvae to light stimuli and distinguished the effects of different genetic contributions. By establishing a standardized method for assessing these behaviors, this study provides a foundation for future research on behavioral development and genetic mechanisms in medaka.

The repetition of the aversive stimulus revealed more pronounced behavioral differences between genotypes, suggesting that the method is effective for exploring stress responses and protective strategies. However, it is important to note the study's limitations, such as the absence of physiological measures and the focus on an early developmental stage, which call for further research.

In conclusion, this research partially confirmed our initial hypothesis that individuals lacking maternal expression of the *auts2a* gene would exhibit fewer anxiety-related behaviors compared to those with maternal expression of the gene. However, it also revealed new insights into the protective strategies adopted based on maternal gene contribution. The results open new avenues for future studies, providing fascinating insights into the evolution of behaviors in response to stressful stimuli and the role of the *auts2a* gene in these mechanisms.

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Résumé

Les mutations du gène *AUTS2* sont associées à des troubles neurodéveloppementaux, dont les Troubles du Spectre Autistique (TSA), mais le rôle de son expression maternelle dans la régulation comportementale est encore mal compris. Cette étude utilise le modèle de larve de médaka (*Oryzias latipes*) pour examiner l'impact de l'expression maternelle du gène *auts2a* sur les réponses comportementales aux stimuli lumineux. Un protocole expérimental a été mis en place pour induire et mesurer ces réponses. Les résultats montrent que l'obscurité est perçue comme un stimulus aversif, entraînant des réactions de stress. Ce protocole a été appliqué à des larves de différents génotypes pour analyser l'effet de l'expression maternelle du gène sur ces comportements. Les larves homozygotes mutantes pour le gène *auts2a* présentent une thigmotaxie réduite. L'absence d'expression maternelle du gène semble induire des stratégies protectrices différentes face aux stimuli aversifs. Ces résultats soulignent l'importance de la contribution maternelle du gène *auts2a* dans la régulation des comportements anxieux et suggèrent que les mutations du gène influencent les mécanismes de réponse au stress. L'étude ouvre des perspectives pour une meilleure compréhension des mécanismes génétiques régulant le comportement.

Abstract

Mutations in the *AUTS2* gene are associated with neurodevelopmental disorders, including Autism Spectrum Disorder (ASD), but the role of its maternal expression in behavioral regulation is still poorly understood. This study utilizes the medaka larva (Oryzias latipes) model to examine the impact of maternal expression of the *auts2a* gene on behavioral responses to light stimuli. An experimental protocol was established to induce and measure these responses. The results show that darkness is perceived as an aversive stimulus, leading to stress reactions. This protocol was applied to larvae of different genotypes to analyze the effect of maternal gene expression on these behaviors. Homozygous mutant larvae for the *auts2a* gene exhibit reduced thigmotaxis. The absence of maternal gene expression appears to induce different protective strategies in response to aversive stimuli. These results highlight the importance of maternal contribution of the *auts2a* gene in the regulation of anxiety-related behaviors and suggest that gene mutations influence stress response mechanisms. The study opens avenues for a better understanding of the genetic mechanisms regulating behavior.