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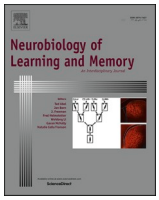

Analysis of neural plasticity genes' expression in fish brain reveals the basis of individual differences in learning

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ABSTRACT

Individual differences in cognition have been historically recognized in humans, and recent evidence suggests that such variation is widespread across the animal kingdom. Despite its importance for individuals' behaviour and fitness, the biological roots of cognitive variation remain poorly understood. We hypothesize that variation in brain gene expression is important in determining individual cognitive differences. To test this, we focused on 6 neural plasticity genes and examined fish, which exhibit the largest cognitive variation reported for vertebrates. Zebrafish (*Danio rerio*) exposed to visual discrimination tasks showed substantial variation in their performance, with some learning over 7 times faster than others. Expression of two genes positively predicted learning performance. However, expression levels of most genes were related at the individual level, suggesting that multi-gene expression patterns may be more relevant than single gene variation. Principal component analysis identified two axes of multi-gene expression variation: the first loaded by all genes except neurotrophin *bdnf*, the second mainly loaded by *bdnf* and *neurod1* expression. Only the latter component significantly predicted learning performance in a visual discrimination task, indicating that individual variation in *bdnf* expression and with lesser extend *neurod1* are critical for learning. Our study bridges the gap between cognitive differences and molecular mechanisms underlying brain function, providing foundation for new understanding what makes individual unique.

1. Introduction

A century of research in psychology has revealed significant individual differences in cognitive performance among humans (Deary 2021; Galton 1870; Thorndike 1918). These differences span a wide range of cognitive domains, encompassing both basic and higher-order functions (Carlson and Moses 2001; Halberda et al. 2008; Kane and Engle 2002; Wilhelm et al. 2010). Evidence of cognitive variability is now emerging across virtually all species investigated, including mammals (Arden and Adams 2016; Beran and Hopkins 2018; Woodley of Menie and Peñaherrera-Aguirre 2022), birds (Ashton et al. 2018; Lambert et al. 2022), non-avian reptiles (Carazo et al. 2014), and teleost fish (Lucon-Xiccato and Bisazza 2017; Lucon-Xiccato et al. 2020), often exhibiting greater variability than that observed in humans. For example, in a fish species, individuals exhibited performance differences of up to 200-fold in an inhibitory control task (Lucon-Xiccato et al. 2020).

The widespread presence of cognitive variation suggests strong biological roots, as well as consequences. Research suggests that cognitive variation has a significant impact on humans' life (Batd 2017; Deary et al. 2010; St Clair-Thompson and Gathercole 2006; Wilens et al. 2011). Similarly, it appears to be a critical factor in animals' survival and reproductive success in nature (Ashton et al 2018; Huebner et al. 2018; Rochais et al. 2023; Smith et al. 2006). Despite this importance and significant research efforts (Friedman et al. 2008; Kanai and Rees 2011; Parasuraman and Jiang 2012; Vogel et al. 2005), the biological bases of cognitive variation remain partly unclear. It is well established that both genetic differences (Davies et al. 2011; Matzel and Sauce 2017) and life experiences (Borsini et al. 2023; Lucon-Xiccato et al. 2023; Pechtel and Pizzagalli 2011; Queller et al. 2023; Sahini et al. 2024) contribute to shaping an individuals' cognitive phenotype. These are also associated to specific patterns of cortical activation in humans

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(Garavan et al. 2006; Hearn et al. 2016; Unterrainer et al. 2004; Waiter et al. 2009). However, the specific molecular mechanisms underlying cognitive variation are still mostly unknown. Part of this gap is due to the challenges of investigating molecular mechanisms in the most studied species, humans, due to ethical constraints.

In this work, we aimed to bridge the gap between cognitive phenotypic variation and molecular mechanisms in the brain by using an emerging research model, the zebrafish *Danio rerio* (Blaser and Vira 2014; Gerlai 2010; Oliveria 2013). We hypothesize that one mechanism at the basis of the cognitive individual differences observed at the phenotypic level could be the variation in the expression of genes in the brain. This hypothesis implies that individuals with differing expression levels of specific genes may exhibit corresponding differences in cognitive performance. Given the preliminary nature of our study and the scarcity of directly comparable researches, a critical first step was to identify genes potentially responsible for individual variability in cognition. We focused on genes involved in neural plasticity, as neural plasticity is widely recognized as a fundamental molecular mechanism underlying learning and memory in mammals (Hölscher, 1990; Mangina & Sokolov, 2006; von Bernhardi et al., 2017). Neural plasticity supports the brain's capacity to adapt and learn by enabling long-term potentiation (LTP), a process that strengthens synaptic connections in response to activity, thereby encoding new information and facilitating memory consolidation (Bliss & Collingridge, 1993; Martin et al., 2000; Goto, 2022). Based on this, we predicted that expression levels of neural plasticity-related genes would be associated with individual differences in cognitive performance. To test this prediction, we selected a panel of genes known from the literature to be involved in neural plasticity and examined whether their expression correlated with learning performance variation in zebrafish.

2. Material and Methods

2.1. Experimental zebrafish

Adult zebrafish subjects (age: 12–18 months; total sample size = 28) were descendants from an outbred wild-type strain bought from a local shop and maintained in the facility at University of Ferrara since 2011. Housing conditions before the experiments consisted of 200-L glass aquaria containing mixed-sex groups of approximately 20 zebrafish. While the subjects were not selected based on sex, previous works found that the groups in the housing were composed by approximately 50 % males and 50 % females. Each aquarium was provided with mechanical, chemical, and biological filters, and water temperature was maintained at 27 ± 1 °C. LED lamps provided illumination with photoperiod set at 14 h: 10 h light: dark. Chemical parameters of water were kept according to the FELASA guidelines (pH range [7–8]; conductivity range [500–1000] μS/cm; $NO_2^- < 0.3$ mg/l, $NO_3^- < 25$ mg/l; DO range [6–8] ppm; Aleström et al., 2020; Tsang et al., 2017). The zebrafish were fed two–three times per day with live nauplii of *Artemia salina* (Artemia Salina Premium GLS, Essen, Belgium) and with commercial flakes (Vipan Nature, Sera, Heinsberg, Germany).

2.2. Learning paradigm

Given that this study exploited an innovative approach, there was no information available on the cognitive abilities more appropriate to investigate the relationship with gene expression. We therefore focused on a simple learning test based on a colour discrimination. The learning test followed a well-established procedure adopted and described in several earlier studies on fish (De Russi et al. 2024; De Russi et al. 2025; Lucon-Xiccato et al. 2022; Lucon-Xiccato et al. 2023; Montalbano et al. 2022). Subjects of the learning experiment (hereafter, 'learner', sample size = 14) were randomly selected from the facility taking care to match their age and size. Each learner subject was then moved individually into an apparatus consisting of a 40 × 25 × 25 cm glass aquarium with natural gravel on the bottom (Fig. 1a), where the entire learning test took place. We built several apparatuses to test all the subjects simultaneously. The illumination was provided by a LED stripe (6000 K; Superlight Technology Co. Ltd., Shenzhen, China) positioned directly above the apparatus with a 14 h: 10 h photoperiod. As the experimental room was kept in darkness and the sides of the apparatus were shielded with green plastic, the subjects were prevented from seeing any external visual cues. Water temperature and other conditions matched those in the maintained tanks. The apparatuses were shaped as an hourglass, with a central corridor that connected the two main sectors facing the short walls of the aquaria (Fig. 1a). Two immature social companions and several natural plants were placed into each lateral compartment at the sides of the central corridor as enrichment. Each day, after the completion of the experiments, the bottom of each aquarium was siphoned to remove faecal material, and new water was added.

The visual stimuli used in the learning test consisted of two discs of identical size (diameter: 1.8 cm, area: 5.65 cm^2), but differing in colour: one yellow and one blue. Each disc was mounted at the centre of a 3 × 3 cm white waterproof plastic card and presented to the subjects using a transparent stick, which could be hung on the short walls of the apparatus. The size of the stimuli was selected based on previous findings: adult zebrafish have an estimated horizontal visual acuity of 1.89 ± 0.02 cycles/degree (Pita et al., 2015), enabling them to distinguish our stimuli from the background at distances up to 54.56 cm. This distance exceeds the total length of the experimental apparatus (40 cm), ensuring that the stimuli were visible to the subjects from any position within the tank. Before commencing the experiment, we assigned to each subject a rewarded colour (either yellow or blue).

The procedure of the learning test consisted of two phases. The first phase was a habituation phase, and started immediately after the subjects were inserted into the experimental apparatus. It lasted three days, during which the subjects could familiarise with the stimuli. In the first day of the habituation phase (day 1), the experimenter administered eight separate trials in which the assigned reward stimulus was inserted into the apparatus. The stimulus was presented directly against one of the short walls of the apparatus. It was inserted while the subject was located on the opposite side of the apparatus. When the subject approached the stimulus within 0.5 body lengths, the experimenter released a drop of water containing live nauplii of *A. salina* as the food reward. The food was released close to the stimulus by means of a Pasteur pipette slowly inserted into the water. The amount of food delivered in each trial was limited to prevent any leftovers. However, the total daily food intake approximately matched the standard maintenance ration to avoid starvation. To maintain the subjects' motivation to perform the task, no food was provided outside the trials. After the subject consumed the food reward and moved away to the opposite part of the apparatus, the experimenter gently removed the stimulus. The eight trials were grouped in two sessions, with four trials in the morning and four in the afternoon. Each trial within a session was separated by a 10-minute interval, either following task completion or when the subject failed to make a choice.

On the two following days of habituation phase (day 2 and day 3), the experimenter administered 12 trials per day in which the two stimuli (i.e., the rewarded colour and the unrewarded colour) were simultaneously presented to the subject. When the subject approached the correct stimulus, the experimenter released the food reward, gently removed the incorrect colour stimulus, and gave the subject 5 min to consume the food. If the subject approached the incorrect colour stimulus, the experiment allowed it to correct its choice before releasing the food and removing the incorrect stimulus. If the subject did not perform a correct choice within 15 min, the experimenter removed the stimuli and repeated the trial later. The left–right position of the two stimuli and the two short walls used to present them were alternated across the trials according to a predetermined pseudorandom scheme to avoid that the subjects learned the visual discrimination task based on the location of stimuli rather than their colour.

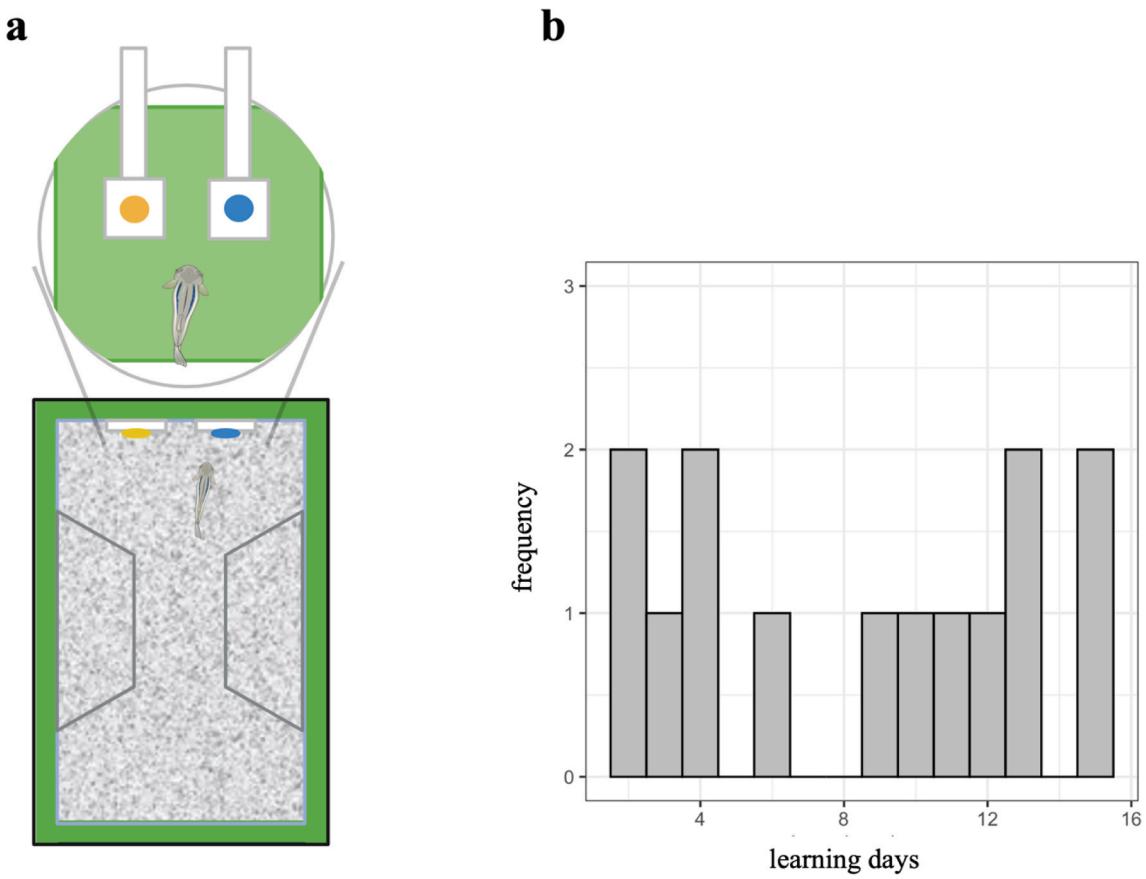

Fig. 1. Learning experiment in zebrafish. (a) Diagram of the experimental apparatus used to study colour discrimination learning abilities in zebrafish. (b) Frequency distribution plot of zebrafish learning performance; the performance is showed as the number of days to reach the learning criterion.

After the three days of the habituation phase, the test phase to score subjects learning performance began. The experimenter administered 12 trials per days with the two stimuli as previously described, but no correction was allowed to the subject's first choice. If the subject approached the incorrect stimulus, the experiment immediately removed the two stimuli and did not administer the food reward. If the subject approached the correct colour stimulus, the experimenter released the food and removed the incorrect stimulus. The learning phase lasted until the subject reached the learning criterion of 17 out of 24 correct trials in two consecutive days. We chose to use a learning criterion to obtain a single data point that would allow us to compare each subject's performance with their gene expression.

2.3. 'Control' subjects' testing conditions

Brain gene expression might be affected by confounding factors such as the exposure to the novel environment (VanElzakker et al. 2008). This might affect our capacity to detect the relationship between gene expression and individual learning performance. We controlled for this potential confound by exposing a second group of zebrafish (hereafter 'control', sample size = 14) to experimental conditions resembling as much as possible those of the 'learner' group. Control subjects matched for age and size the ones of the 'learner' group. Testing took place in the same apparatuses previously described. The procedure also followed that described for the 'learner' group, but 'control' subjects did not receive the training to associate the stimulus with a food reward. After the habituation phase, 'control' subjects were exposed to 12 pseudo trials per day. In each pseudo trial, the experimenter presented the colour stimuli as described before. When the subject approached one of the two stimuli, the experimenter gently removed one of the stimuli from the apparatus but did not administered food. The lack of food reward during the trials ensured no temporal contingency between the stimuli and the reward, and therefore no possibility to learn. However, to match as much as possible the experience of the experimental subjects, the food ratio of each control subject was delivered one hour before and one hour after the daily sessions of trials. Each control subject was randomly assigned to a 'learner' subject and exposed to the pseudo trials for the same number of days.

2.4. Brain dissection and Real Time PCR

Thirty-minutes after a 'learner' subject reached the learning criterion, we euthanised it in a bath containing water and MS222 (Tricaine methanesulfonate; Sigma, Germany). A 'control' subject was euthanised at the corresponding day of the pseudo learning experiment. Because the 'control' subjects had the same experience of the 'learner' subjects, but had no chance to learn, eventual differences in gene expression between these two groups should be due to baseline individual differences that may affect learning (VanElzakker et al. 2008). Under a dissection microscope, we collected the whole brain from each subject and stored it in a 1.5 ml laboratory tube containing Trizol™ reagent (ThermoFisher Scientific, Italy). We pestled the brain tissue to obtain a homogenous solution and then stored at −20 °C. After collecting the brain tissue from all the 'learner' and 'control' subjects, we proceeded with RNA extraction using TRizol™ reagent (ThermoFisher Scientific, Italy) following the manufacturer's instructions. The extraction was performed separately per each subject to obtain individual levels of gene expression. The amount, quality and purity of isolated RNA were analysed by BioSpec-nano (Shimadzu Italia, Italy). DNase-treated RNA (1U for 1 μg of RNA) was used to synthetize cDNA using iScript™ c58DNA Synthesis Kit (Bio-Rad Laboratories, Italy) and a thermocycler (CFX Connect Real-Time PCR Detection System, Bio-Rad Laboratories, Italy). The

quantitative PCR of the final volume of 10 µl was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Italy) in triplicate on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Italy) instrument.

To quantify the expression of a panel of target genes involved in learning processes and neuroplasticity (*bdnf*, *neurod2*, *shisa7b*, *neto1l*, *neurod1*, *grin1a*) (Chen et al. 2017; Cunha et al. 2010; Gould et al. 2019; Lin et al. 2005; Lucon-Xiccato et al. 2022; Ng et al. 2009; Salvanes et al. 2013; Schmitz et al. 2017; Sengar et al. 2019; Wilke et al. 2012), we used primers indicated in Table 1 (D'Agostino et al. 2022; Lee et al. 2018; Morbiato et al. 2019; Paiva et al. 2020). The raw data of *bdnf* expression have been used in an earlier control experiment (Lucon-Xiccato et al. 2022). As housekeeping genes, we used *rpl13a* and *18S*, based on their validation in similar experimental settings (Morbiato et al. 2019; Lucon-Xiccato et al. 2024). To assess their stability across our experimental conditions, we checked standard deviation of Cq values across all samples, which ranged from 0.81 to 0.93. These values fall within the commonly accepted threshold (SD < 1), indicating consistent expression. For these reasons, we considered these genes appropriate internal controls for our expression analysis. We verified the efficiency of the primers by constructing standard curves for all genes investigated. Moreover, the dissociation curve was used to confirm the specificity of the amplicon. The relative levels of expression of each sample were calculated by the $2^{-\Delta\Delta CT}$ method, where CT is the cycle number at which the signal reaches the threshold of detection (Livak and Schmittgen 2001). The geometric mean of two housekeeping genes (*rpl13a* and *18 s*) has been used for the normalization (Table 1). Each CT value used for these calculations is the mean of three replicates of the same reaction.

2.5. Statistical analyses

All statistical analyses were conducted in RStudio version 4.2.1 (2022–06-23 ucrt; http://www.rstudio.com/). We first conducted an analysis to exclude that the number of subjects that achieved the learning criterion was not due to the chance. The learning performance expected by chance was simulated by settings at 50 % the probability of an individual to choose the correct response in 12 binomial trials, corresponding to the daily number of trials administered to each subject during the experiment. The learning performance was simulated for up to 15 sets of trials, which corresponded to the observed maximum number of days that a subject took to reach the criterion. We replicated the simulation 10,000 times, and then, we computed the percentage of subjects that achieved the learning criterion in each simulation. We calculated the probability of observed successful subjects in the population of simulated random performance (De Russi et al. 2024). A second simulation was performed to evaluate the average number of days required for 14 fish to achieve the learning criterion by chance. By using the same approach for simulating the expected performance by chance, we ran 10,000 replicates to estimate the average performance of a group of 14 fish in terms of number of days to criterion.

We also analysed the cognitive performance of the 'learner' group as the decreased number of errors across the training. The data consisted in the total number of correct choices and the number of errors recorded per each daily session. This data was combined into a two-column matrix using the "cbind" function from the "base" R library; then this was used as the response variable in a Generalised Linear Mixed-effects Models (GLMMs) with binomial error distribution using the "glmer" function from the "lme4" R library. The model was fitted with day as covariate, subject ID as random effect to account for the repeated-measurements structure of data. The significance of effect on the response variable was tested via the "Anova" function from the "car" R library. We also evaluated whether individual differences in the learning performances could be attributed to the cognitive performance on the first day of training using a Spearman rank correlation test.

To address the question of whether individual variance on expression in our set of neuroplasticity genes might determine the cognitive performance in the 'learner' group, we used a set of separate Spearman rank Correlation tests. Each of these tests evaluated a separate of hypothesis, whether a specific gene predicted learning performance, resulting in independent testing (Althouse, 2016; Hooper, 2025). In these tests, learning performance was the numbers of days to achieve the criterion.

Because the exposure to the experimental setup might affect the expression of neural plasticity genes, we performed the same rank-based correlation tests on the 'control' group. After finding this hypothesized significant relationship, we decided to further explore the link between gene expression and learning accounting for this factor. To do so, we calculated an index for each gene of the 'learner' group that was corrected for confounding factors not related to learning. First, we computed a linear regression between the day of exposition to the testing apparatus and the expression of each neural plasticity gene in the 'control' group. Then, we extracted the predicted values for each gene from the regression line with respect to the day of exposition to the apparatus (Fig. S3). By using the predicted values of genes' expression, we calculated the scart of gene expression of the 'learner' group from the predicted value of 'control' group as following:

$$\text{index} = \frac{\Delta\text{gene expression}_{\text{'learn' group}} - \text{predicted value of 'control' group}}{\text{predicted value of 'control group'}}$$

This new index was used in the following analysis as individual level of gene expression corrected from the influence of factors not related to learning such as the exposure to the apparatus. For instance, 'learner' subjects with positive scart for a certain gene with respect to the predicted value from the regression line of the 'control' subjects had higher than average gene expression, and *vice versa*.

We performed pairwise correlation analyses using Pearson product–moment Correlation tests on the new set of indices to assess the presence of within individual variation and whether gene expression predicted cognitive performance. We performed a Bonferroni multiple-testing correction using the "corr.test" function form "psych" R library. We found that most of the pairwise correlations on the new index showed significant positive value, suggesting that pattern of genes expression rather than single genes might determine individual differences in learning. This covariation among the expression of the target genes also prevented the use of a single model to predict learning due to collinearity. Therefore, we used the Principal Component Analysis (PCA), a multivariate technique that allow to reduce a high number of correlated variables into few uncorrelated principal components, which maximise the variance present in the original dataset (Budaev 2010;

Table 1
Forward and reverse primers used in the PCRs.

Genes	Forward primer	Reverse primer	Source
bdnf	ATAGTAACGAACAGGATGG	GCTCAGTCATGGGAGTCC	D'Agostino et al 2022
neurod2	CCTTGGCGACGAGGACTTAG	TTTGGGTCTGTCTCCATCACC	Lee et al 2018
shisa7b	CAAACCAATGACCACCAGCA	TGGCACCATCCCACCATAAT	Primer3 https://primer3.ut.ee/
neto1L	GCGTAATTATGTGGCGGTGT	TCTTTCTGCTGCCCTCATCA	Primer3 https://primer3.ut.ee/
neurod1	CATGCTACCCCTCCATGTTAAT	CATTAGACGGGATTCAGAGGAC	Lee et al 2018
grin1a	CACCAGGATGTCCATTTATTCA	CCTTAGGTCCTCTCTTGTTGTCA	Paiva et al 2020
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	Morbiato et al 2019
18 s	ACCACCCACAGAATCGAGAAA	GCCTGCGGCTTAATTTGACT	Morbiato et al 2019

Johnson & Wichern, 2002). Importantly, this method allowed us to simultaneously study the effect of all the predictors (e.g., expression level of the different genes) in a single regression model. Before computing the PCA, the indices of two genes, i.e. *neurod1* and *grin1a*, were log-transformed due to right-skewed distributions. The PCA was performed on the standardized index of genes' expression by using the "prcomp" function from "psych" R package (https://cran.r-project.org/web/packages/psych/index.html). For a better graphical representation of results, the loadings and the scores (i.e., the coordinates of each individual on the principal components) were multiply by −1 thanks to the symmetry properties of the resulted components. Spearman rank correlations were calculated to assess the relationship of each component to the learning performance.

3. Results and discussion

3.1. Individual zebrafish remarkably differed in learning performance

All the zebrafish of the learner group achieved the learning criterion within two weeks. On average, the zebrafish took 8.5 ± 4.9 days to reach the learning criterion, with notable individual variance (range: 2–15 days; Fig. 1b): slow learners required up to seven times more training to master the task than fast learners. The observed success in learning the task (100 % successful subjects) was a considerably higher than expected by chance (computer-generated simulation of successful subjects expected by chance within 15 days of training: 31.82 ± 12.45 %; comparison between simulated results and observed data: $P < 0.001$; Fig. S1a). Notably, a second simulation revealed that the simulated subjects achieved the criterion in 38.28 ± 9.77 days, which corresponded to more than twice the maximum number of days took by the real subjects (Fig. S1b). In general, these simulations suggested that the learning performance of our zebrafish was unlikely due to chance. While we cannot completely rule out the possibility that some subjects reached the learning criterion due to a few random choices, this is not expected to affect our main results for two reasons. First, subjects who could have met the criterion by chance were typically those already performing well in the task. Second, it is equally likely that some subjects failed to reach the criterion on a given day due to one or a few random errors.

A repeated measures analysis revealed a significant decrease in the number of errors made by the subjects across the days of the learning test (GLMM: ß = −0.084, $\chi^2_1 = 25.275$, $P < 0.001$, marginal $R^2 = 0.182$; Fig. S2), further supporting that zebrafish learned the discrimination task. The model also revealed that a relevant proportion of explained variance could be ascribed to the individual differences (adjusted Interclass Correlation Coefficient: 0.432; AIC of null model without random effect 490.54, AIC of model with random effect: 477.47; likelihood ratio test of random effects term: $\chi^2_1 = 15.067$, $P < 0.001$). When we tested for a relationship between the first day performance and the number of days required to achieve the learning criterion, we found a significant negative correlation (Spearman's rank correlation: $\rho = -0.766$, $IC95^{th} = [-0.94;-0.35]$, $S = 803.69$, $p = 0.001$), indicating that individuals with higher cognitive performance on the first days learned faster the visual discrimination task.

Our findings corroborate earlier reports of exceptionally high cognitive variation in fish relative to other vertebrate taxa (Lucon-Xiccato et al., 2020). We propose that such pronounced individual differences could have important evolutionary consequences. Although direct evidence in fish remains scarce (Smith et al., 2006; De Russi et al., 2024), studies in other vertebrates indicate that cognitive variation can influence fitness (Ashton et al., 2018; Cole et al., 2012; Huebner et al., 2018; Rochais et al., 2023). If cognitive performance affects survival or reproduction, selection should act on these traits, provided they possess sufficient heritable genetic variation. Because laboratory-reared fish are raised under uniform conditions, at least part of the observed variation is likely genetic. Consequently, given the large cognitive variance observed in fish, selection on cognitive abilities in this group may be particularly effective, potentially driving rapid adaptive evolution. Future work should test this prediction, for example by comparing responses to artificial selection on cognitive traits in fish versus other vertebrate groups.

3.2. Raw expression levels of single neural plasticity genes did not explain learning differences

The expression of neural plasticity genes was not significantly related to the number of days required to reach the learning criterion (Fig. 2a). This result appeared to contradict our hypothesis. However, the exposition to the novel environment during the learning experiments might have altered brain gene expression (VanElzakker et al. 2008), potentially confounding the relationship of gene expression with learning performance. We addressed this issue by evaluating variation in brain gene expression in the 'control' group of zebrafish exposed to the same environmental condition of the first 'learner' group. Neural plasticity genes were generally more expressed in the 'control' subjects that spent more days in the testing apparatus (Fig. 2b), although this trend was fully significant only for *bdnf* and *neto1l*, and marginally significant for *shisa7b*. These results support our hypothesis that exposure to the apparatus itself affects the expression of neural plasticity genes.

3.3. Controlled indices of single genes expression predicted learning

To account for the confounding effect of the exposure to the apparatus, we conducted a follow-up correlation analysis using a new set of gene expression indices corrected for this factor. Two out of 6 expression indices (i.e., *bdnf* and *shisa7b*) significantly predicted learning (Fig. 3a; Fig. S3). This supports our hypothesis that differential expression of neural plasticity genes contributes to cognitive individual difference. Note, that, to support an earlier study on a mutant zebrafish line lacking a functional *bdnf* gene, we had already analysed the relationship between *bdnf* expression and learning in our subjects, with findings comparable to those of the present study (Lucon-Xiccato et al., 2022). While no current studies in other vertebrates can be directly compared to our results, they are partially supported by literature employing different approaches. For instance, post-mortem examinations in humans have shown that the expression of certain genes in the brain predicts cognitive decline (Buchman et al. 2016). However, other similar studies found no relationship between brain gene expression and intelligence measures (Gosso et al. 2007). In laboratory animal models, several studies have reported that experiential manipulations such as acute stress or environmental enrichment can simultaneously alter brain gene expression and cognitive performance (Gosso et al. 2007; Hüttenrauch et al. 2016; Reshetnikov et al. 2018). It is worth noting that the causal link between learning and gene expression often remains speculative. Within this framework, the novelty of our study lies in providing direct evidence that brain gene expression predicts individual cognitive performance, at least in zebrafish. We expect that this relationship may also hold true for other vertebrates. However, to test this, it is necessary to adopt a similar experimental design and conduct comparable studies across different species.

3.4. Indices of single genes expression were highly correlated at the individual level

When we examined individual differences in the expression of neural plasticity genes with a correlation approach, we found that 11 out of 15 pairwise comparisons showed moderate to high, positive covariation ($r_s = 0.40 – 0.81$; Fig. 3b; Supplementary Table 1). The covariation was significant for 8 gene pairs: *neurod1* vs *neurod2*; *neurod1* vs *grin1a*; *neurod1* vs *neto1l*; *neurod2* vs *neto1l*; *neurod2* vs *grin1a*; *neto1l* vs *grin1a*; *neto1l* vs *shisa7d*; *grin1a* vs *shisa7d*. Six of these relationships remain significant even after correcting for multiple comparisons. This finding indicates that some individuals consistently exhibited higher expression

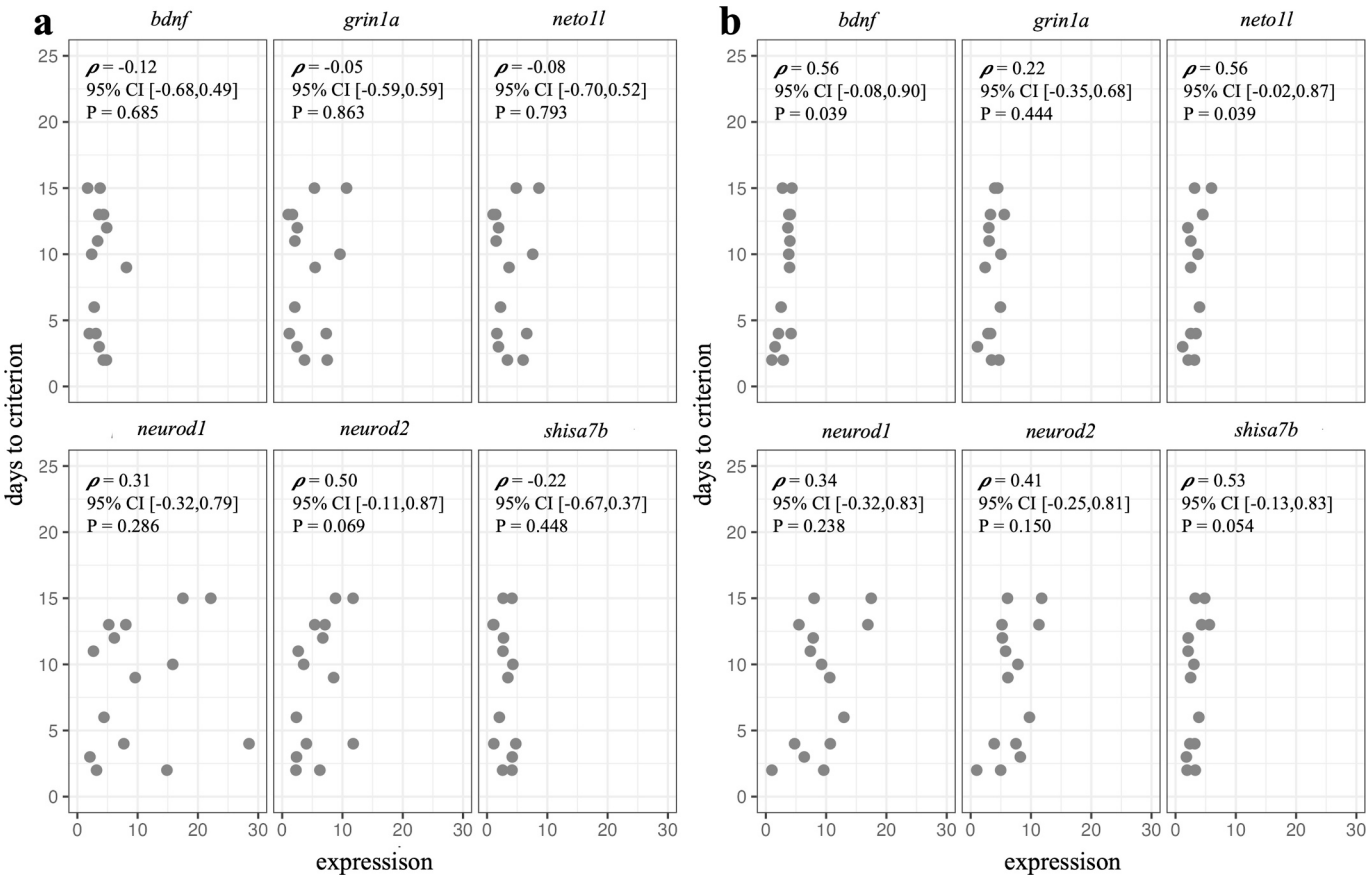

Fig. 2. Analyses of the relationship between the raw gene expression and day of training in zebrafish. (a) Scatterplots showing the relationship between the number of days to achieve the learning criterion and the expression level of single target genes in the 'learner' zebrafish. (b) Scatterplots of the number of days to achieve the learning criterion versus the expression level of one of the single gene analysed in the 'control' group of zebrafish. In each panel, the Spearman's rank coefficient, the estimated 95% confidential interval, and the significance are reported.

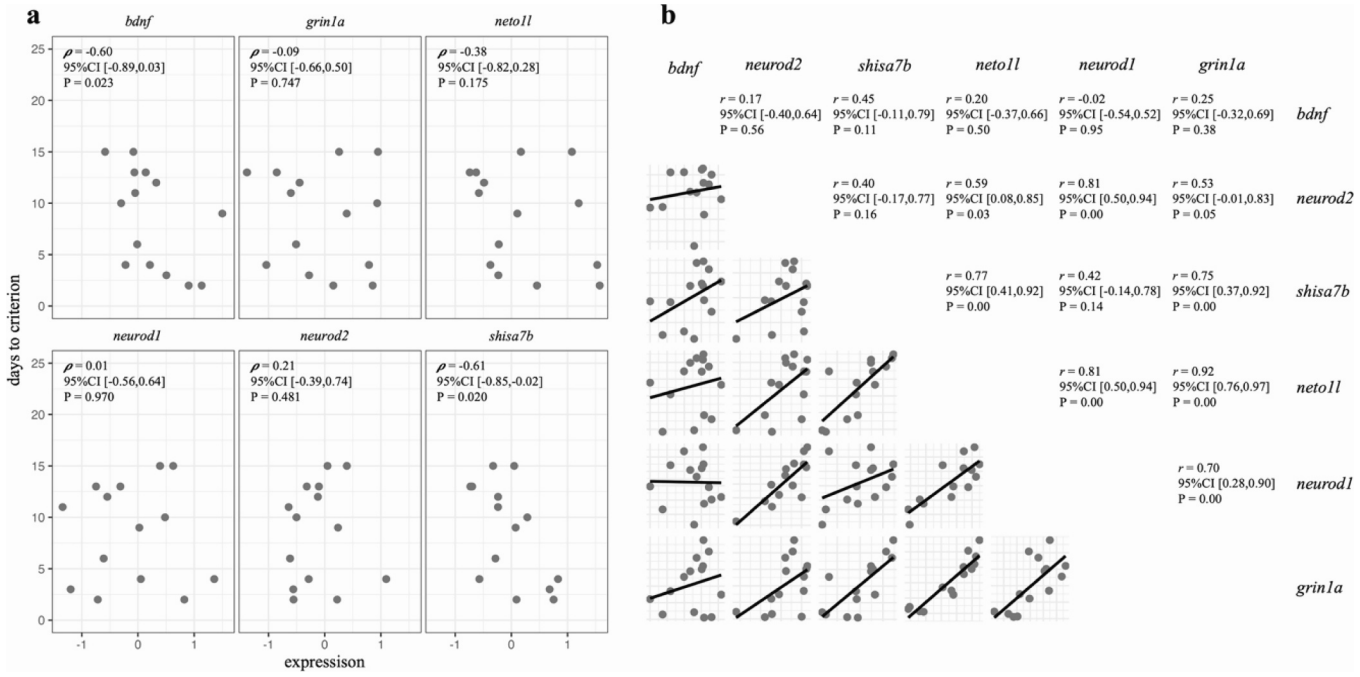

Fig. 3. Analyses of the indexes of gene expression corrected with the 'control' group of zebrafish. (a) Linear relationship between the number of days to achieve the learning criterion and the index of expression of the target genes; in each panel, the Spearman's rank coefficient, the estimated 95% confidential interval, and the and significance are reported. (b) Scatterplots and Pearson's correlation matrix of the covariance among the indexes of brain gene expression.

levels across multiple neural plasticity genes. Individual differences in neural plasticity genes expression are therefore not fully captured by examining genes in isolation. Conversely, they may reflect broader patterns of co-expression involving gene networks (McKenzie et al. 2018). This insight prompted us to apply multivariate techniques to better understand the relationship between multi-gene expression profiles and individual learning performance.

3.5. *Multivariate analyses of gene expression revealed critical predictors of learning*

Different parameters of sampling adequacy suggested suitability of the panel of neuroplasticity genes for factor analysis. The Kaiser-Meyer-Olkin value was 0.63 and the internal consistency of the correlation matrix was $\omega = 0.87$ $IC95^{th}$ [0.77–0.96]. Moreover, the predictors showed high level of multicollinearity (Variance Inflation Factor, VIFs > 1.50) and the Bartlett's test of sphericity was significant ($\chi^2_{15} = 62.264$, $p < 0.001$) (Budaev 2010; Johnson & Wichern, 2002). We therefore run a Principal Component Analysis (PCA) finding that the expression of the different genes clustered around two regions (Fig. 4a; Supplementary Table S2). The PCA analysis confirmed that the two components with eigenvalues > 1 explained 82.77 % of total variance in neural plasticity gene expression. The first principal component (PC1) explained 63.09 % of the total variance. The second principal component (PC2) explained 19.68 % of the total variance. All the genes (loading factors > 0.39) but *bdnf* (loading 0.16) had a relevant impact on PC1 (Fig. 4b). Conversely, PC2 was mostly loaded by *bdnf* (loadings 0.77) and *neurod1* (loading −0.42) with an opposite direction, with a minor contribution from *shisa7b* (loading −0.39: Fig. 4b; Supplementary Table S2).

Multiple regression with the component scores revealed that PC2 was a significant predictor of learning performance (Spearman rank Correlation: $\rho = -0.576$, $IC95^{th} = [-0.90; -0.04]$, $S = 717.15$, $p = 0.031$; Fig. 4c) but PC1 was not ($\rho = -0.258$, $IC95^{th} = [-0.75;0.31]$, $S = 572.52$, $p = 0.373$; Fig. 4d). Because PC2 was mostly loaded by *bdnf* and in the opposite diretion, by *neurod1*, we conclude that these two genes exerted the greatest influence on learning. Zebrafish with higher expression of *bdnf* and lower expression of *neurod1* in the brain were faster at learning a colour discrimination task.

The role of the *neurod* family on cognitive variance is mostly unknown. Recent studies in humans have linked *neurod2* mutations to various neurological conditions, including autism spectrum disorders (Runge et al. 2020b) and pathophysiology of neurocognitive dysfunctions (Spellmann et al. 2017). Mouse models with *neurod2* mutations exhibited impaired emotional learning capacities (Lin et al. 2005), resembling those symptoms observed in human diagnosed with autism. Because the key role of the *neurod* family in the development, function and survival of the central nervous system is shared among several vertebrates (Dokucu et al. 1996; Liao et al. 1999; Olson et al. 2001), we might expect its involvement in cognitive variation, but the mechanism is still unknown. Conversely, the pivotal importance of *bdnf* for learning aligns with our previous work on a zebrafish mutant line. Knockouts

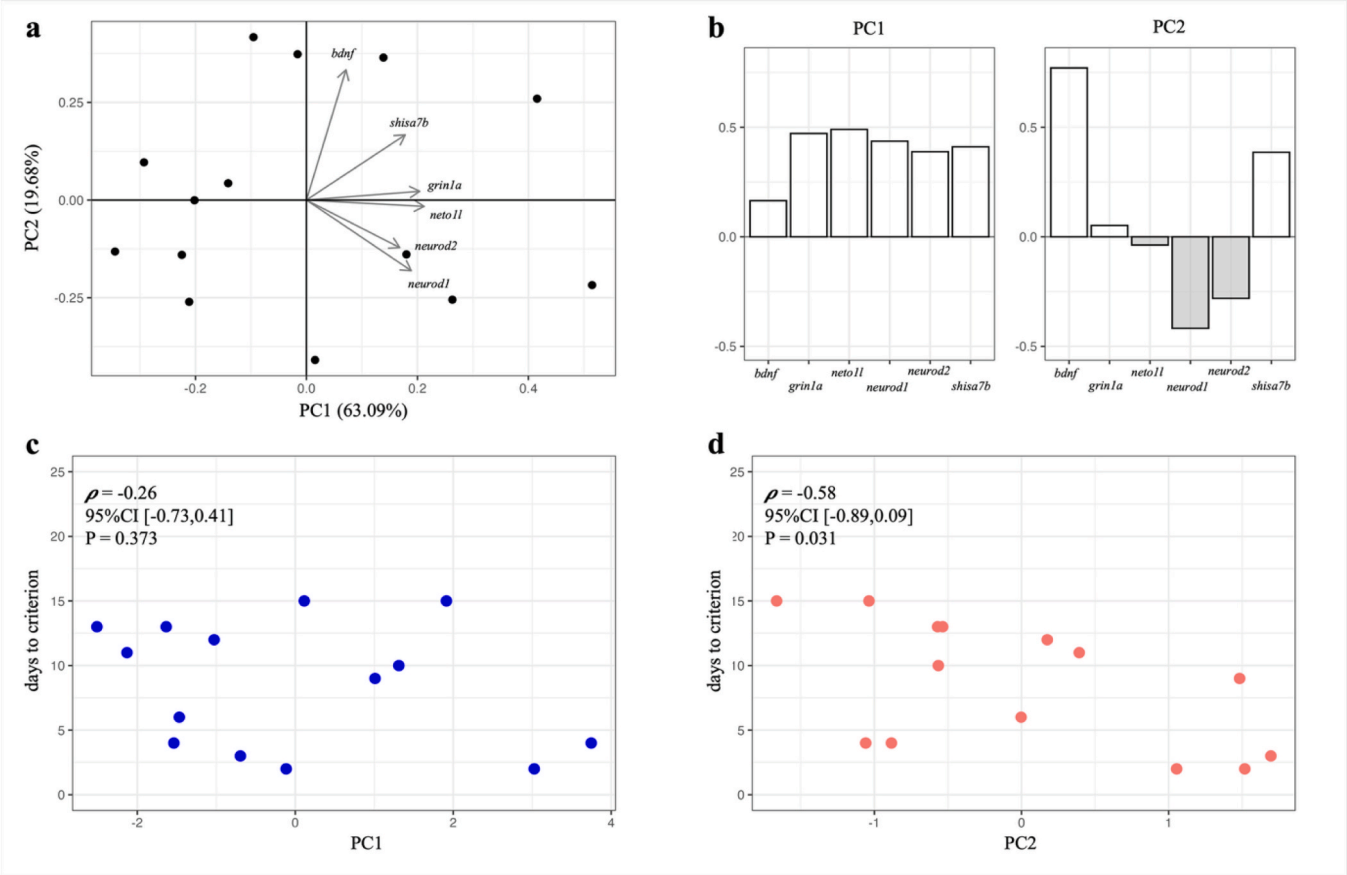

Fig. 4. Multivariate analyses of neural plasticity genes expression and learning in zebrafish. (a) Biplot of the two components with eigenvalue greater than 1 obtained from the PCA; the arrows indicate the rotation of each gene to the components. (b) Contribution (loading) of each learning index to the variance explained by the two components obtained from the PCA. The colour bar indicated the positive (white) or negative (grey) contribution of each learning index to the components. (c, d) Relationship between the first (blue colour) or second component (red colour) with the number of days to criterion; the Spearman rank coefficient, the estimated 95% confidential interval, and the and statistical test are reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lacking *bdnf* displayed substantial cognitive deficits such as slow learning rates and poor executive functions (Lucon-Xiccato et al. 2022). Additionally, previous studies support the relevance of *bdnf* in other species (Linnarsson et al. 1997; Lucon-Xiccato et al. 2022; Salvanes et al. 2013; Silakarma and Sudewi 2019; Yamada et al. 2002). For example, exposure to environmental enrichment and physical exercise has been shown to simultaneously increase BDNF levels in the brain of mammalian models and enhance learning and memory performance (Fan et al., 2016; Kazlauckas et al., 2011; Novkovic et al., 2015; Xu et al., 2011; Wang et al., 2019). However, it is important to note that these findings provide only indirect evidence, as they do not isolate BDNF's role in cognitive differences among individuals reared under uniform conditions, as our study does. It should also be noted that *bdnf* influences a wide range of gene pathways (Gottschalk, 1999; Kowiański et al., 2018), as well as the transcription of the *neurod* family involved in neuronal differentiation (Tutokova et al., 2021), suggesting that some of its effects on learning may result from indirect mechanisms.

bdnf has a relatively conserved sequence across vertebrates (Hallböök 1990; Tettamanti et al. 2010). The evidence of similar effects of *bdnf* on learning across species suggests that the fundamental mechanisms underlying this cognitive ability may have evolved early in the history of this clade. One may even speculate that the role of *bdnf* in mammals' learning is greater than in zebrafish. Indeed, the mutant mice model lacking this gene display alterations so severe that is not vital (Ernfors and Lee 1994). On the other hand, due to genomic duplication events, teleost fish possess 3 different neurotrophins (Hallböök 1990; Hallböök et al. 2006; Lanave et al. 2007). Their contribution on cognitive individual differences deserves attention.

4. Conclusions

This study sheds light on the molecular bases underlying the cognitive variance observed across vertebrates (Arden and Adams 2018; Ashton et al. 2018; Beran and Hopkins 2018; Carazo et al. 2014; Lambert et al. 2022; Lucon-Xiccato and Bisazza 2017; Lucon-Xiccato et al. 2020; Woodley of Menie and Peñaherrera-Aguirre 2022). Expression levels of neural plasticity genes predicted individuals' learning abilities in zebrafish. In the light of the conserved nature of brain molecular mechanisms across vertebrates (Cunha et al. 2010), our findings in zebrafish are likely relevant for the entire clade, including humans.

We focussed on neural plasticity genes because they are main actors in the molecular and cellular processes underlying learning (Chen et al. 2017; Cunha et al. 2010; Gould et al. 2019; Lin et al. 2005; Lucon-Xiccato et al. 2022; Ng et al. 2009; Salvanes et al. 2013; Schmitz et al. 2017; Sengar et al. 2019; Wilke et al. 2012). However, genes with other functions also deserve attention. The results of our study are indeed influenced by the specific genes investigated, and we cannot exclude the possibility that including additional genes in the analysis might reveal the importance of other genes not identified in our work. Importantly, the substantial covariation observed between the expression of the genes analysed suggested that multi-gene analyses are crucial for understanding the molecular bases of learning.

While our study addresses proximate genetic mechanisms driving individual differences in learning, it also raises stimulating research questions. For instance, could gene expression explain cognitive sex differences? Although such differences appear rare in zebrafish (Roy & Bhat, 2017), they are common in other fish and non-fish vertebrates (reviewed in Jonasson, 2005; Jones et al., 2003; Lucon-Xiccato, 2022). Moreover, what determines individual variation in the expression of cognition-relevant genes? Is it primarily genetic makeup (Hing et al., 2018), environmental influences shaping gene expression via phenotypic plasticity (Novkovic et al., 2015), or both? Dedicated research is needed to disentangle these contributions.

5. Declaration of AI use

We have not used AI-assisted technologies in creating this article.

6. Data and code availability

All datasets used in this study are provided as a supplemental information. All code generated during this study is provided as a supplemental information. Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Elia Gatto (elia.gatto@unife.it).

Ethics statement

The research was approved by the Institutional Animal Care and Use Committees of the University of Ferrara (protocol no. TLX_1-2020) and by the Italian Ministry of Health (auth. no. 340/2019-PR). The experiments were conducted in accordance with the ARRIVE guidelines (du Sert et al. 2020). License for zebrafish maintenance and breeding at the facility of University of Ferrara is no. 29/2023-UT.

CRediT authorship contribution statement

Elia Gatto: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elisa Samorì:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Elena Frigato:** Writing – review & editing, Methodology, Investigation, Data curation. **Cristiano Bertolucci:** Writing – review & editing, Writing – original draft, Resources, Methodology, Data curation, Conceptualization. **Tyrone Lucon-Xiccato:** Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2025.108106.

Data availability

All datasets and R code used in this study are provided as a supple-

mental information.

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