

Research article

Knockout in zebrafish reveals the role of the glucocorticoid receptor in shaping behavioral syndromes

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ABSTRACT

Glucocorticoids (GCs) have a wide spectrum of effects on animal behavior. A recently suggested effect involves determining the structure of individual differences, that is how the behavioral traits of an individual covary, forming the so-called behavioral syndromes. As GCs can exert their action in multiple ways, e.g., via rapid non-genomic effects or via the activation of two highly homologous members of the steroid receptor family acting as transcription factors, it is unclear how the GC modulation of behavioral syndromes takes place. We exploited a zebrafish line with a frameshift mutation in the gene encoding the GC receptor (Gr), to investigate this question. We found that lack of Gr altered the average score of several behavioral traits in the mutant line, determining reduced boldness, and increased activity and sociability. Critically, the pattern of covariation between these traits was also substantially affected by the loss of Gr. The most evident effect was an association of traits involved in boldness in the *gr* mutant line. This study reveals that, in zebrafish, Gr is not only involved in the modulation of the average value of behavioral traits, but also in how the behavioral traits of an individual are interrelated and determine the behavioral syndromes.

1. Introduction

Glucocorticoids (GCs) are steroid hormones involved in the adaptive plasticity processes that adjust the phenotype to environmental variations in, for instance, resource abundance, climate, competition, and predation risk [39,81,102]. The effects of GCs are broad [81], encompassing changes in glucose metabolism [26,30,42], rhythmic expression of clock-controlled genes [26,64], immune response [33,34], development [66,73], reproduction [29,56], homeostasis restoration after stress [39,42], as well as in behavior [26,39,40,70,72].

Regarding behavior, the levels of circulating GCs typically vary between individuals (reviewed in [22]); thus, these hormones are not surprisingly involved in individual behavioral differences (e.g., [6,13–16,31,74]). In particular, the level of GCs has been linked to the structure of covariation among different behavioral traits within individuals, the so-called behavioral syndromes [3,18,91]. These are critical features of animal behavior, found in virtually all species investigated and often linked with fitness [17,34,85].

How GCs affect behavioral syndromes is still an open question

because of the different potential action mechanisms. Besides the non-genomic effects that GCs mainly exert through the interaction with cytosolic or membrane-bound receptors [88], GCs can also cross cell membranes and regulate transcription of target genes upon the activation of two different intracellular steroid receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) [80]. Mr and Gr have evolved from a cyclostome (jawless fish) ancestral corticoid receptor (CR) through gene duplications, leading to the first appearance of Mr and Gr as distinct orthologues in cartilaginous fishes [4]. One correlative study in wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) found a behavioral syndrome between boldness and aggressiveness and additionally reported that both traits were positively correlated with brain expression of Gr, suggesting that GCs and Gr are the mechanistic basis of behavioral syndromes [3]. Moreover, Mr display a tenfold stronger affinity for GCs, causing Gr activation to occur following stress-induced peaks in GCs levels [54,81]. The fact that the stress response of GCs is often reported to predict individual behavioral differences [6,21] still points towards the implication of Gr in behavioral syndromes.

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In this study, we experimentally tested the hypothesis that the effects of GCs on behavioral syndromes involve Gr. We exploited a zebrafish (*Danio rerio*) knockout (KO) mutant line ($gr^{ia30/ia30}$) obtained through a CRISPR/Cas9 approach [28]. The zebrafish is a teleost fish, but differently from most teleosts that present two *gr* paralogous genes resulting from a fish-specific whole-genome duplication event [62], this species has the additional advantage of a single copy gene (*nr3c1*; [1]). Furthermore, *gr* KO zebrafish are viable [28], in contrast to the mouse null line for this receptor [23], which die perinatally due to defects in lung maturation. In zebrafish, Gr functions have been analyzed by knockdown approach and by generation of *gr* mutant zebrafish lines (reviewed in [24]), some with reported alterations in single behavioral traits [26,105].

In our study, we administered a battery of four established behavioral tests to the knockout zebrafish and control wild-type siblings: i) the open-field test that provides estimates of activity levels, boldness, and exploratory behavior in novel environments [12,53,58,105]; ii) the diving test that investigates boldness based on swimming depth [27,58]; iii) the scototaxis test that analyzes the preference for dark environments displayed by many teleosts as an indicator of shyness [12,58,59]; and iv) the shoaling test that measures variation in fish sociability [58]. We then analyzed the structure of covariation (syndrome) of the observed behavioral traits and compared it between the two genotypes.

2. Materials and methods

2.1. Ethics statement

Husbandry and experimental procedures were carried out in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and the Italian (D.lgs. 26/2014) animal protection standards. Research was approved by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health (auth. num. 128/2020-PR).

2.2. Experimental fish

Forty-nine zebrafish were used in the experiment, 34 of the zebrafish null $gr^{ia30/ia30}$ mutant line [28], hereafter referred to as $gr^{-/-}$, and 15 wild-type siblings ($gr^{+/+}$) as the control group. Wild-type lines used for the generation of the stable *gr* mutant line included Tuebingen, Giotto and Umbria strains [71]. Fish from both genotypes were one year old. The number of subjects assayed depended on the availability of fish with the same age in the facility. We chose to test all available subjects to increase the statistical power as much as possible.

Before the experiments, the fish were kept in the facility of the University of Ferrara, in multiple 200 L glass tanks (115 × 40 × 50 cm), divided per genotype. The sex ratio in the maintenance tanks was approximately 50:50. By using all available fish we ensured a balanced number of males and females between the two genotypes. The facility had 14:10 h light:dark cycle. The water in the tanks was kept with the following parameters: temperature 27 ± 1 °C; conductivity 606.7 ± 60.18 µS/cm; nitrite below 0.1 mg/L; and nitrate below 50 mg/L. Each tank was equipped with an aerator and mechanical, chemical, and biological filters. The fish were fed twice per day with newly hatched nauplii of *Artemia salina* and dry food (Staple food Vipan, Sera, Heinsberg, Germany).

2.3. Experimental design

Each subject underwent the four tests sequentially during the same day. This experimental design was chosen because it allowed us to easily recognize each individual and therefore, to study individual differences as for the scope of this study. If the tests were administered with longer intervals, the need to identify the individuals would require invasive marking techniques or social isolation, which are both known to affect

zebrafish behavior (e.g., [32,78,84]). The tests were administered following a fixed order. This strategy is recommended to study individual differences because it avoids different carry-over effects across individuals [8]. The order of the tests was chosen considering that two of them (i.e., open-field test and diving test) were related to the response evoked by novelty. Therefore, to ensure strong responses, we first administered these two tests to the subjects.

On the day of testing, the subjects received food one hour before the experiment begun. This was done to avoid the confounding effects of hunger-motivated behaviors during the test. For instance, in other fish species, hunger determines an increase in activity [37,99]. In all the tests subjects from the two strains, randomly selected from the maintenance tanks, were tested alternately (two $gr^{-/-}$, one $gr^{+/+}$). Tests took place between h 09:30 and 17:00.

2.4. Open-field test

The open-field test was conducted according to a common procedure for teleost fish [53,89]. Each subject was individually moved in an unfamiliar environment consisting of a white, empty arena (40 × 40 cm) (Fig. 1A). The water depth was 8 cm and its temperature was 27 ± 1 °C. The open-field arena was uniformly illuminated (15 lux) from above by warm-white LED strips (Superlight Technology Co. Ltd., Shenzhen, China). The arena was kept in a room separated from the facility to minimize external disturbance.

After releasing the subject in the center of the arena, the experimenter started a camera placed above the apparatus (Monochrome GigE camera, Basler, Germany). The subjects' behavior was then recorded for 20 minutes. As the open-field provides indicators based on the response evoked by an exposure to a novel environment [27], the recording started immediately after the subjects were transferred. This allowed to include in the analyses the initial response of the subjects before they showed habituation to the novel environment. The recordings were conducted in infrared light spectrum exploiting an infrared backlit table ($\lambda > 980$ nm; Noldus Information Technology, Wageningen, The Netherlands) placed under the arena. The subject position was tracked automatically by the EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands), which then computed two variables describing the reaction to the novel environment. The first variable was a distance moved, used as a measure of activity. The second variable was the time spent in the center of the arena indicating boldness (at least 1 body length, approx. 3 cm, from the edge of the arena). Authors from different disciplines often describe this variable differently, considering it either a measure of boldness or anxiety-like behavior. In our work, we will operatively use the term boldness because boldness is the trait typically considered in research on behavioral syndromes [3,86,100]. We cannot exclude that our findings could involve what is normally referred to as anxiety-like behavior. Individuals displaying lower boldness (shier individuals) were expected to spend more time close to the edge of the arena (thigmotaxis; [20,58,105]). To handle cases in which the software failed to track the position of the fish for few seconds, the variables were corrected for the available tracking time obtaining proportion variables (e.g., distance moved over tracking time and time spent in the edges over tracking time).

2.5. Diving test

The diving test was used to record zebrafish typical behavior of swimming close to the bottom of the environment when stressed [26,27,58]. The experimental subject was transferred from the open-field arena to a 20 × 20 × 20 cm tank (water depth: 18 cm; water temperature: 27 ± 1 °C) (Fig. 1B). The diving tank was illuminated by a warm-white LED strip (265 lux; Superlight Technology Co. Ltd., Shenzhen, China) placed on the top of the apparatus. The tank had three white walls and one transparent wall. The transparent wall was used to record the position of the fish in the water column by means of a camera (HDR-CX405, Sony

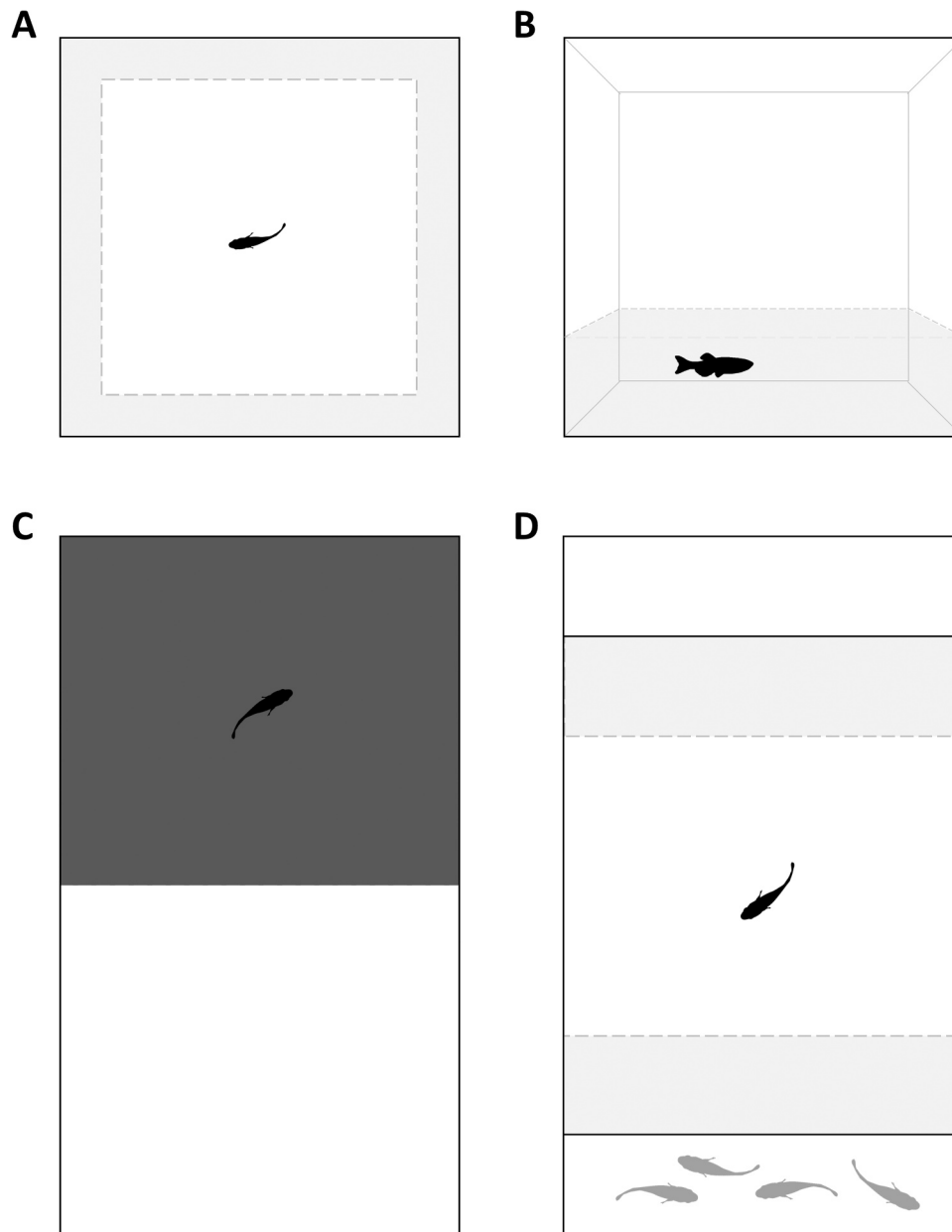


Fig. 1. Schematic view of the set-up of the four experiments. (A) Open field test arena view from above. (B) Vertical exploration tank view from the side. (C) Scototaxis test arena view from above. (D) Sociability test set-up view from above. Grey areas bounded by dashed lines in (A), (B), and (D) represent the areas used to score fish behavior.

Europe B.V., Weybridge, UK) placed on a tripod. The recording lasted 20 minutes. The subject position was, as for the open-field test, tracked automatically by the EthoVision XT software (Noldus Information Technology, Wageningen, The Netherlands). We measured shyness as time spent in the lower quarter of the water column, within 4,5 cm from the bottom as the threshold. Shier individuals were expected to spend more time close to the bottom [27,50]. As for the open-field test, this variable is considered by some authors as a measure of anxiety.

2.6. Scototaxis test

Scototaxis, defined as the preference for dark environments over lighted ones, is a common shyness-related behavior of teleosts [12,59]. This behavior is considered an adaptive strategy for predator avoidance [59]. The set-up used for this test consisted of a $56 \times 15 \times 32$ cm tank (water depth: 8 cm; water temperature: 27 ± 1 °C), in which the subject

was transferred after completing the diving test. The walls and bottom of one half of the apparatus were covered in white plastic, while the other half was covered in black plastic (Fig. 1C). The apparatus was illuminated by warm-white fluorescent lamps (320 lux). The subject was free to move in both areas, and its behavior was video recorded (HDR-CX405, Sony Europe B.V., Weybridge, UK) for 20 min from above. The behavior was then scored from the video recordings using the custom software Ciclic timer (v. 1.3). This software consisted of a set of independent stopwatches that could be activated with the computer keyboard. The experimenter that scored the recordings was blind with respect to the genotype of the subjects. The software allowed to compute the time spent by each fish in the black sector of the arena. Shier individuals, or more anxious ones according to some authors, were expected to spend less time in the white sector of the apparatus. We did not use automatic tracking software for this test due to difficulties in tracking the subjects across substrates of different color (i.e., white and

black).

2.7. Shoaling test

Our test measured shoaling tendency as the time spent close to a social stimulus [19]. For this test the subject was moved to the central compartment of a three-chamber apparatus. The apparatus was overall $60 \times 20 \times 36$ cm (water depth: 8 cm; water temperature: 27 ± 1 °C; Fig. 1D). The partition between the central and the lateral compartments (15×20 cm each) was made of transparent plastic. Four zebrafish unfamiliar to the experimental subjects were placed in one of the lateral

compartments as social stimulus, while the other compartment remained empty. The apparatus was illuminated in correspondence of the lateral chambers to ensure visibility of the stimuli by warm-white fluorescent lamps (108.5 lux). The subject was video recorded (HDR-CX405, Sony Europe B.V., Weybridge, UK) from above for 20 min. Then, the recordings were analyzed using Ciclic timer (1.3) to calculate the time spent close (1 body length) to the compartment with the social stimulus and the time spent close to the empty compartment. Subjects with higher sociability were expected to spend more time close to the social stimulus.

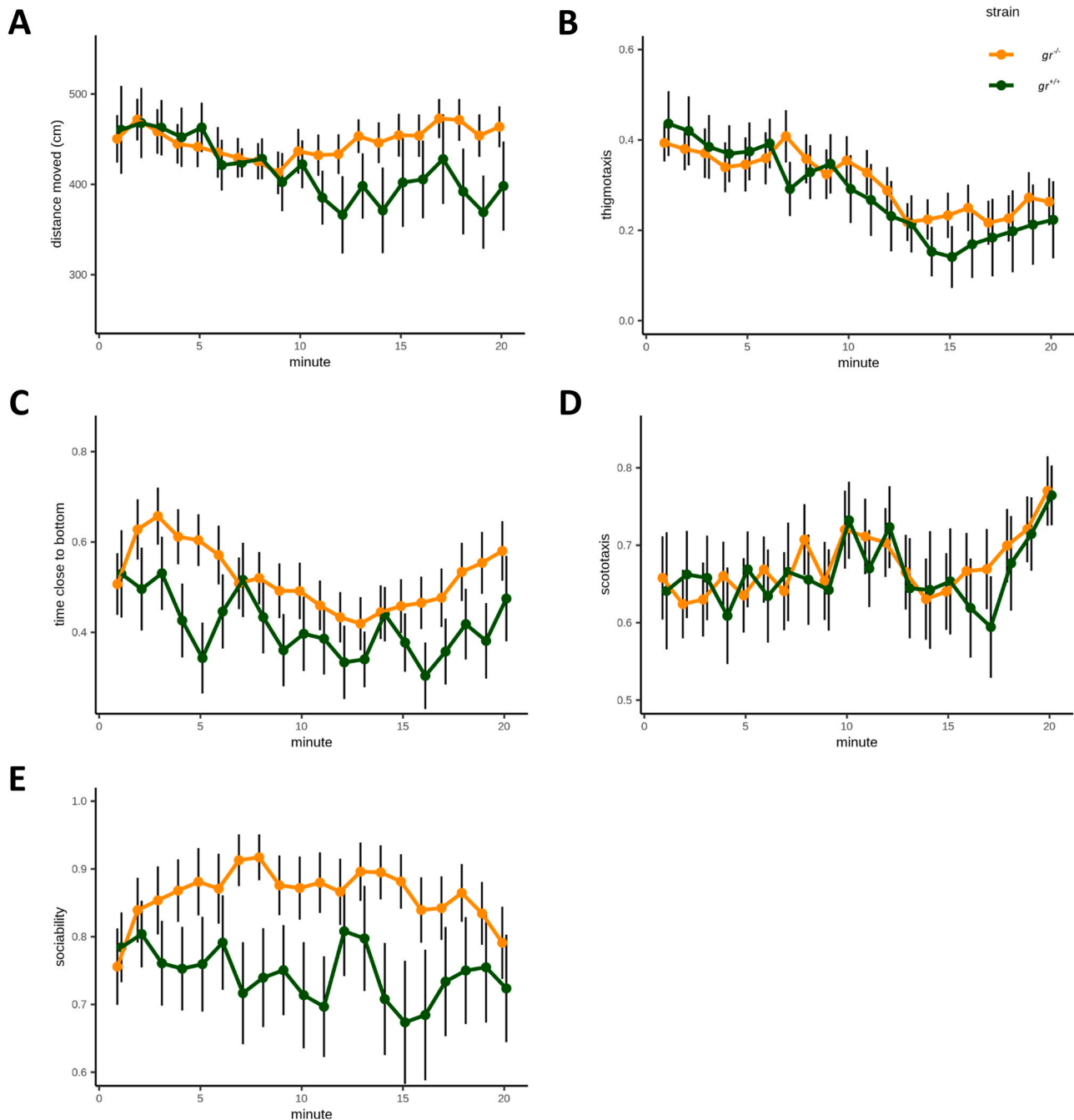


Fig. 2. Comparison between the two zebrafish genotypes ($gr^{+/+}$, $N = 15$; and $gr^{-/-}$, $N = 34$) in the average values of the behavioral traits collected. (A) Activity of the subjects as distance moved in the open-field test. (B) Thigmotaxis as proportion of time spent close to the edge of the arena in the open-field test. (C) Time close to the bottom as a measure of shyness, reported as proportion of time spent in the lower quarter of the tank in the diving test. (D) Scototaxis as proportion of time spent in the black area of the scototaxis apparatus. (E) Sociability as proportion of time spent close to the shoal in the sociability test. Data points represent means and error bars represent standard errors.

2.8. Statistical analysis

All statistical analyses were done in R version 4.2.2 (<https://www.R-project.org/>). Significance was set at $P < 0.05$. Experimental data were first analyzed using ANOVAs to test for significant average differences between the two strains. In these analyses, we used repeated measures models with the behavioral data split in the 20 minutes of test as the dependent variable. This allowed us to investigate variation in the response to the novel environment, which is typically stronger at the beginning of the test. We further analyzed the average differences between strains using the machine learning (ML) approach described by Mamede and colleagues (2024). The ML function was trained with a random subsample (70 % of the subjects) to recognize the difference between the two strains. Using the 'rpart' function, we generated a decision tree displaying the behaviors most effective at discerning between zebrafish with and without *gr*. Subsequently, ML's accuracy was tested by observing its performance in predicting the strain of the remaining subjects.

Thereafter, we studied the correlation between behavioral traits. First, we run a Pearson correlation analysis between pairwise combination of traits, divided per each genotype. For this correlation analysis we excluded two subjects (one *gr*^{+/+} and one *gr*^{-/-}) as they were missing data from the open field and the shoaling test, respectively, due to logistic issues. Last, we performed a Principal Component Analyses (PCA) per each genotype using the 'prcomp' R function, which allows to summarize the information contained in a data set with multiple inter-correlated quantitative variables and to express this information as a set of new variables (Principal Components).

3. Results

3.1. Open field test

In the open-field test, the average distance moved by the zebrafish during the 20 minutes was 8760.23 (\pm 1935.19, SD) cm. We found a significant effect of the strain on the distance moved (repeated measures ANOVA: $F_{954} = 15.84$, $P < 0.001$), with the *gr*^{-/-} being more active than the *gr*^{+/+} (Fig. 2A). Additionally, we found the interaction between strain and time to be significant ($F_{954} = 11.21$, $P < 0.001$), as well as the effect of time ($F_{954} = 27.03$, $P < 0.001$). Regarding our measure of boldness, the zebrafish spent 29.40 (\pm 18.88) % of the time close to the tank walls. We did not find a significant difference in the time spent by the two strains close to the area edges (repeated measures ANOVA: $F_{954} = 1.686$, $P > 0.05$; Fig. 2B), nor a significant effect of the time on thigmotactic behavior or the interaction between the two strains ($F_{954} = 0.273$, $P > 0.05$; $F_{954} = 2.250$, $P > 0.05$).

3.2. Diving test

In the diving test, the zebrafish spent on average 49.0 (\pm 27.01) % of the time in the lower quarter of the tank. The *gr*^{-/-} zebrafish spent significantly more time in the lower quarter of the tank as indicated by a significant effect of genotype (repeated measures ANOVA: $F_{974} = 23.878$, $P < 0.001$; Fig. 2C). We found no significant effect of time on fish behavior ($F_{974} = 2.536$, $P > 0.05$) and no interaction between time and strain ($F_{974} = 0.008$, $P > 0.05$).

3.3. Scototaxis test

On average, the zebrafish spent 67.06 (\pm 17.46) % of the time in the black area of the apparatus. This suggests the presence of the scototaxis, a typical behavioral trait of the species. When we compared the two genotypes, we found no significant difference (repeated measures ANOVA: $F_{974} = 0.489$, $P > 0.05$) in the preference for the dark area between the two experimental groups (Fig. 2D). Time had no effect on the scototaxis behavior displayed and the interaction between the two

variables was not significant ($F_{974} = 0.832$, $P > 0.05$; $F_{974} = 0.208$, $P > 0.05$).

3.4. Shoaling test

In the shoaling test, the subjects spent on average 82.27 (\pm 22.40) % of the time close to the social stimulus. We observed a significant effect of the strain on the fish shoaling tendency, with *gr*^{-/-} spending significantly more time close to the shoal (repeated measures ANOVA: $F_{954} = 39.047$, $P < 0.001$; Fig. 2E). The effect of time and the interaction were not significant ($F_{954} = 0.219$, $P > 0.05$; $F_{954} = 0.708$, $P > 0.05$).

3.5. Machine learning analyses

The ML approach confirmed that the two strain could be identified based on average behavioral differences. The most relevant variable in the decision tree was the time spent in the bottom of the diving test, which was higher in *gr*^{-/-} subjects (Fig. 3). Thigmotaxis was an additional variable important for the discrimination. The accuracy of prediction was 73.33 %.

3.6. Correlations between behavioral traits

The correlation tests revealed evidence of covariation between various behavioral traits (Fig. 4). However, the pattern of covariation was different between the two genotypes. In the *gr*^{+/+} subjects, there were no significant correlations (Fig. 4A). In the *gr*^{-/-} subjects, there were two significant correlations (Fig. 4B): time spent in the lower quarter in the diving test correlated with both scototaxis ($\rho_{31} = 0.420$, $P = 0.015$) and thigmotaxis in the open-field test ($\rho_{31} = -0.710$, $P < 0.001$). An additional correlation analysis was conducted considering only the latter 10 minutes of the activity in the open-field test, because in this interval we found a significant effect of the strain. The new correlation analysis revealed an additional significant correlation in the *gr*^{-/-} subjects (activity in open-field versus thigmotaxis: $\rho_{31} = -0.423$, $P = 0.014$).

3.7. Principal components analysis

In both *gr*^{+/+} and *gr*^{-/-} zebrafish, the PCA analysis identified three principal components (PC) that collectively explained most (i.e., approximately 80 %) of behavioral traits' variance (Table 1). In line with what observed in the correlation analysis, the PCA identified different relationships between behavioral traits in the two strains (summarized in Fig. 5 and Table 2). For instance, PC1 was mainly loaded by thigmotaxis and sociability in *gr*^{+/+} subjects, while in *gr*^{-/-} subjects it was loaded by a cluster of traits related to boldness (thigmotaxis, scototaxis, and time spent close to the bottom). PC3 was loaded by activity and boldness traits in *gr*^{+/+} subjects, while it was almost exclusively loaded by activity in *gr*^{-/-} subjects.

4. Discussion

Previous studies showed that GCs is involved in the modulation of behavioral syndromes, i.e., correlation between different behavioral traits. In this study, we asked whether Gr contributes to such modulation. To this goal, we observed mutant zebrafish lacking a functional *gr* coding gene in four standard behavioral tests: open-field, diving, scototaxis, and sociability tests. Our results indicate that the structure of covariation between behavioral traits is altered by the lack of Gr. Moreover, *gr* loss determined an average increase in activity and sociability, and a decrease in one of the boldness parameters.

Significant evidence of pairwise correlations between different behavioral traits was found only in the *gr*^{-/-} zebrafish. In particular, the significant correlations concerned the three traits related to boldness: the thigmotaxis in the open-field test, the scototaxis, and the time spent

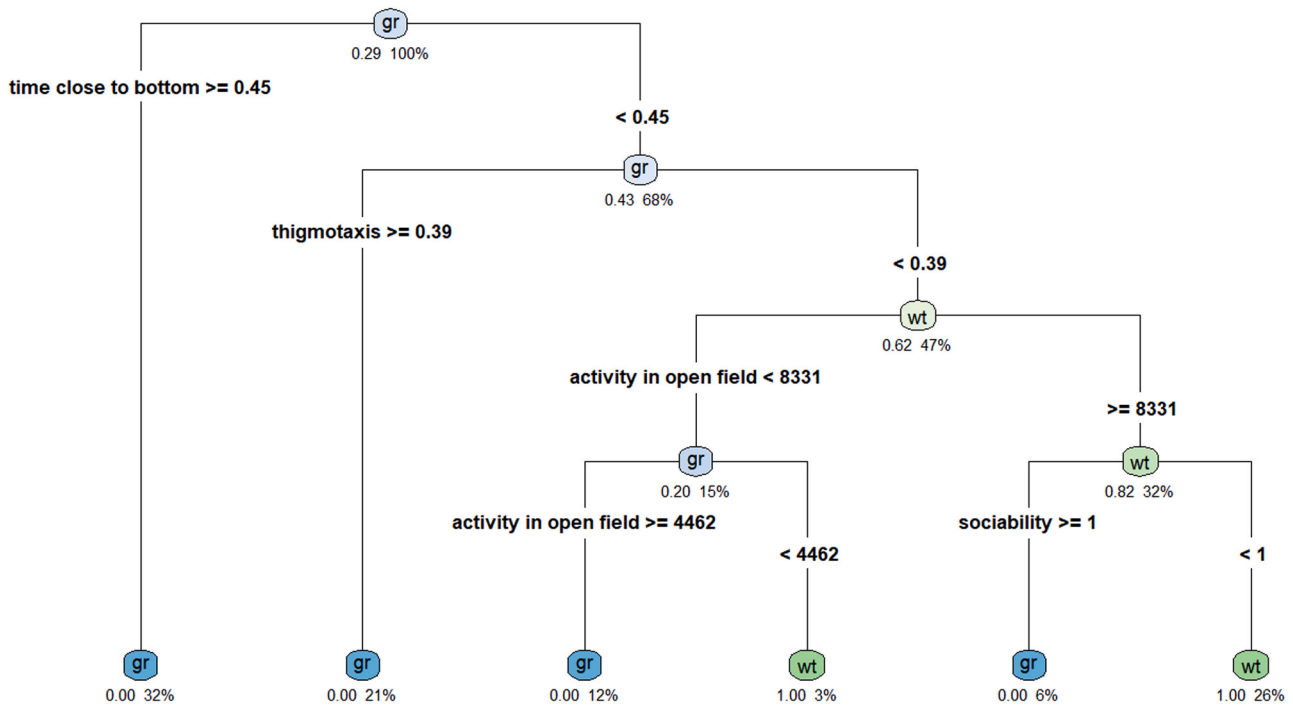


Fig. 3. Decision tree for strain prediction. Percentages of individuals reaching each node are reported under each node. Branches split all the individuals entering the model following rules based on the behavioral variable measures, reported on each branch (e.g., time close to bottom ≥ 0.45). Numbers below each node indicate the Gini Index.

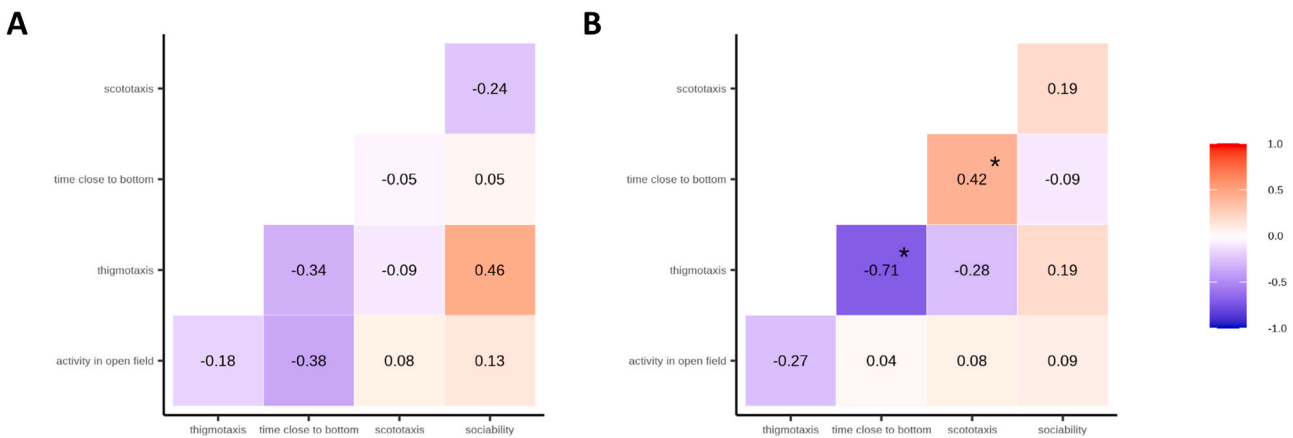


Fig. 4. Correlation matrices between the behavioral traits of (A) $gr^{+/+}$ zebrafish (N = 15) and (B) $gr^{-/-}$ zebrafish (N = 34). Asterisks denote statistically significant correlations (* $P < 0.05$). ρ values are plotted with representative color scale.

Table 1

Individual and cumulative contribution to behavioral traits variance of the three principal components identified in each genotype by PCA.

Genotype	PC1	PC2	PC3	Cumulative
$gr^{+/+}$	32.19 %	27.72 %	19.74 %	79.75 %
$gr^{-/-}$	40.42 %	23.76 %	19.84 %	84.02 %

close to the bottom in the diving test. Notably, there were no trends for the same correlations in the $gr^{+/+}$ zebrafish, suggesting that this finding was not due to different statistical power between the two genotypes. Furthermore, the component explaining the greater proportion of behavioral variance identified by the PCA analyses was loaded by a cluster of boldness traits only in $gr^{-/-}$ zebrafish. We conclude that zebrafish lacking Gr showed stronger covariation between boldness

traits. Gr is therefore involved in the mechanism underlying boldness-related behavioral syndromes and, specifically, in the pathway determining their lessening. In one of the significant correlations in $gr^{-/-}$ zebrafish (time close to the bottom versus thigmotaxis), the direction of the relationship also suggested the idea of consistency between indicators measuring the same trait. Accordingly, it is worth investigating whether gr loss also affect consistency of behavioral differences.

This finding on the syndrome with boldness traits is apparently consistent with studies in a range of species reporting a link between behavioral traits and physiological measures of stress [2,47,57,69]. In particular, Bell and Sih [9] reported the emergence of a boldness/aggressiveness syndrome in three-spined sticklebacks exposed to predation risk. Killen and colleagues (2013) provided evidence that under stressful conditions, covariation between traits might strengthen. Additionally, Guenther et al. [36] experimentally demonstrated that cortisol increases the strength of correlation between boldness and docility in the cavy,

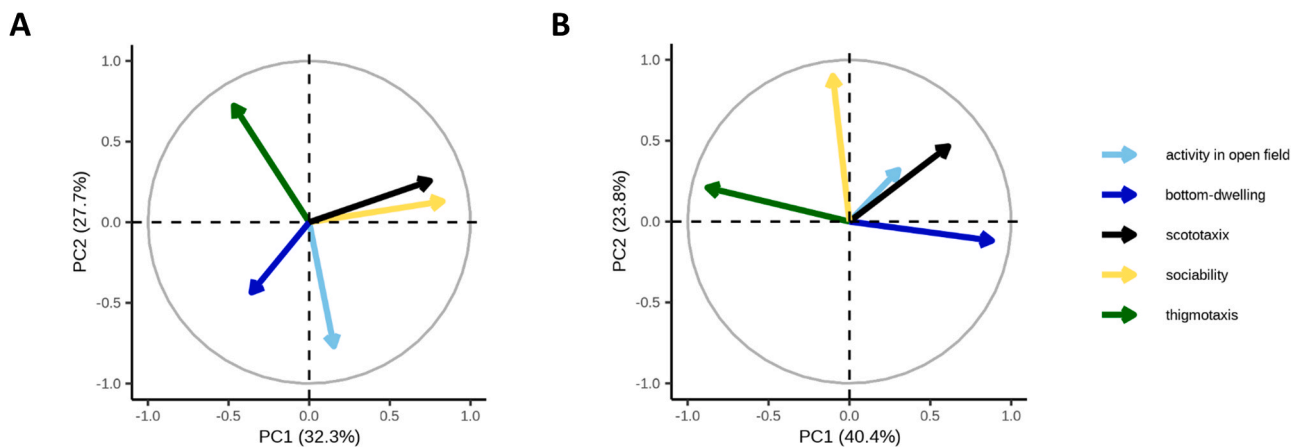


Fig. 5. Variable factor maps obtained from the PCA of the behavioral traits of (A) $gr^{+/+}$ and (B) $gr^{-/-}$ zebrafish ($N = 15$ and $N = 34$, respectively). Each plot is drawn on a factorial plane, the vector space created by the intersection of the two principal components (PCs). Correlations between a variable and a PC are used as the coordinates of the variables on the PC (positive correlations group variables together while negative ones position the variables on opposite quadrants). Angles of 90° between variables indicate no correlation. The length of the arrows represents how well the variance of the data is explained by the variables on the factorial plane. Numbers between parenthesis indicated percentage of variance explained by each component.

Table 2

Summary of the principal component analysis results. With each factor are listed variables with the respective loadings. Highlighted in bold are the major positive and the major negative loadings on each factor (major loadings are defined as a bivariate correlation coefficient between the variable and the factor with an absolute value greater than 0.40).

Behavioral trait	PC1 $gr^{+/+}$	PC1 $gr^{-/-}$	PC2 $gr^{+/+}$	PC2 $gr^{-/-}$	PC3 $gr^{+/+}$	PC3 $gr^{-/-}$
Activity	0.120	0.213	-0.653	0.293	0.556	-0.882
Thigmotaxis	0.651	-0.617	0.110	0.193	-0.481	0.157
Time close to bottom	-0.368	0.619	0.614	-0.108	0.234	0.214
Scototaxis	-0.281	0.430	-0.370	0.427	-0.564	0.374
Sociability	0.589	-0.072	0.218	0.827	0.294	0.112

Cavia aperea. These records suggest that strong behavioral syndromes involving boldness and similar traits emerge under stressful conditions due to the action of GCs. Our findings suggest that Gr helps lessen the GC-mediated syndrome via its negative feedback effects [10], after the end of a stressful event. It would now be interesting to evaluate the eventual contribution of Mr to this effect.

The emergence of behavioral syndromes under naturally occurring stress, such as predation risk, has been often interpreted as adaptive [9]. For instance, it may reflect trade-offs between different antipredator strategies such as avoidance and inspection [25,38,41,45,48,55,60,61]. We argue that also the lessen of such covariation is adaptive, when requested by the context. Indeed, natural populations from different habitats often display variability in the structure of covariation [77]. Accordingly, the role of Gr in lessening the syndrome is complementary to that of other eventual GC pathways in strengthening them. Another potential adaptive role of the GR pathway acting on the boldness syndrome can be related to the timing of the stressful event. Timing of the onset of a stressor with respect to the circadian cycle can greatly influence the HPA response to stress [46]. Studies on natural populations described how the response to predation stress during the day is modulated adaptively (e.g., [63]). In a study on the mutant line of the present work, Morbiato et al. [64] demonstrated a critical role of *gr* on circadian regulation. It is worth investigating if this action of *gr* is related to the adaptive circadian variation in response to stressors.

While the most evident difference between $gr^{+/+}$ and $gr^{-/-}$ zebrafish was related to the aforementioned boldness traits, the PCA analyses also suggested other changes in the structure of behavioral syndromes, which can be visualized by plotting the components scores on a variable factor

map (Fig. 5). For instance, in $gr^{-/-}$ zebrafish, sociability and activity appeared isolated in PC2 and PC3, while in $gr^{+/+}$ they were assigned to variance components significantly loaded also by other traits (Table 2). We can therefore conclude that Gr is involved in the action exerted by GCs on the behavioral syndromes between all the traits analyzed in this study. Notably, additional traits not measured in this study, such as aggression (e.g., [44,65]) and antipredator responses [103], are often part of behavioral syndromes. It is possible that also these other traits are under the influence of Gr signaling. Moreover, the traits investigated in our work can be measured with different tests. For example, we measured response to novelty by presenting an unfamiliar environment, but the same could be done by presenting novel objects [49]. We can therefore expect that also results of these tests should be susceptible to Gr's effects. Additionally, an interesting direction for future studies is examining the interplay between GCs signaling, behavioral covariations, and metabolism [104], which could be at the bases of the observed modulation of Gr on activity.

The conclusion that Gr is involved in the pathway through which GCs alter behavioral syndromes is in line with the hypothesis behind this work. Individual differences are usually predicted by the stress-related increase of GCs [6,21]. This increase is expected to activate Gr, which otherwise would be outcompeted by the stronger affinity of Mr for GCs [54,81]. Moreover, one experimental work has already presented correlative data linking Gr expression and behavioral syndromes [3]. This does not exclude the involvement of other pathways. For instance, even if not supported by preliminary data, the involvement of Mr is still possible. Gr controls a negative feedback loop that reduces circulating GCs [82]. Therefore, mutant zebrafish lacking Gr may also show alterations in circulating GCs and Mr activation, both possibly involved in behavioral syndromes.

Regarding the differences in the average behavior between the two genotypes, our first finding is that $gr^{-/-}$ zebrafish were more active than the $gr^{+/+}$ zebrafish in the open-field test. An increased locomotor activity was observed in Gr mutant mice when compared to the controls [7, 67]. However, the effect in the present study depended on the testing time. In the initial minutes of the test, the two strains did not differ in their activity, suggesting a similar initial response to the novel environment. In the following ten minutes, the control group displayed approximately 9 % less distance moved. Therefore, the difference between the two genotypes emerged when looking at the change in activity between the initial and the second half of the test. Accordingly, it was found that gr^{357} mutant zebrafish did not habituate to a novel tank after repeated exposure [105]. In mammals, GR signaling has been associated

to cognitive abilities including learning [52,95]. As habituation is considered a form of non-associative learning [11], also in zebrafish Gr might be involved in cognition. Alternatively, considering that erratically swimming in the arena is considered by some authors as an indicator of anxiety [20,58], we cannot exclude that the effect on activity was related to differences between genotypes in anxiety-traits. This alternative interpretation of the activity data aligns with the findings of the diving test, in which the $gr^{-/-}$ zebrafish spent more time than $gr^{+/+}$ zebrafish near the bottom of the apparatus. The preference for the bottom of the tank is a behavior that has been ascribed to shyness in zebrafish [58,90]. Therefore, in our study, the $gr^{+/+}$ zebrafish appeared to be bolder as they spent significantly less time in the lower quarter of the tank. Our result on boldness supports previous findings in zebrafish with the diving test: Eachus and colleagues (2023) reported that gr^{s357} null mutants made fewer entries compared to controls in the upper area of the apparatus. Notably, individual differences in behaviors related to boldness have important consequences. For instance, fitness in natural populations is related to the individual levels of boldness [5,101] and bolder individuals may display lower plasticity levels [43]. These consequences suggest the importance to study the indirect effects exerted by Gr-mediated alterations of behavioral interindividual differences.

Interestingly, we found no significant differences between the two genotypes in another measure related to boldness, the thigmotaxis, as it was already found in heterozygous and homozygous mice for *Gr* [68,76,79]. Conversely, three earlier studies on zebrafish gr^{s357} mutants have reported changes in thigmotaxis. Ziv and colleagues [105] and Eachus and colleagues [26] found that these mutants showed a decreased thigmotaxis, while Sireeni et al. [87] found the opposite result. Given these findings, we can reasonably conclude that *gr* is involved in the regulation of traits such as boldness and anxiety. However, the specific direction of the effect and the eventual parameters (including methodological ones) modulating such responses remain unclear. Part of the contrasts between the different studies can be due to the fact that our line is a null *gr* mutant, whereas in the gr^{s357} line the gene encoding Gr presents a missense mutation in the DNA binding domain that allows it to maintain some residual transcriptional activity not involving DNA-binding but rather the interaction with other transcription factors [35].

Lastly, results from our sociability test showed that the $gr^{-/-}$ mutants have a greater shoaling tendency compared to $gr^{+/+}$ zebrafish. Evidences suggest a role of GCs in sociability [75], as it was demonstrated that continuous increases in GCs levels can affect social behavior. Notably, the greater sociability shown by the $gr^{-/-}$ can be linked, at least in part, to predator avoidance [83,93,92,94,96,98], since shoaling is generally used as a strategy to reduce predation risk [51,97]. Thus, $gr^{-/-}$ zebrafish higher sociability can be related to the reduction in boldness discussed above.

In conclusion, the use of a zebrafish mutant line lacking the Gr unveiled the role of this receptor pathway on behavioral syndromes. Our study concurs in highlighting that the zebrafish has an emerging role as a model to study GCs' effects in vertebrate behavior [35,87,82]. This role not only applies to zebrafish biology, but also possesses high translational potential [27,82,105].

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CRedit authorship contribution statement

Tyrone Lucon-Xiccato: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Cristiano Bertolucci:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Luisa Dalla Valle:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Francesca Terrin:** Writing – review & editing. **Eleonora Rovegno:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Conflict of interest

We declare no conflict of interest.

Data Availability

Data will be made available on request.

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