Does the stress axis mediate behavioural flexibility in a social cichlid*, Neolamprologus pulcher*?

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Abstract

Behavioural flexibility plays a major role in the way animals cope with novel situations, and physiological stress responses are adaptive and highly efficient mechanisms to cope with unpredictable events. Previous studies investigating the role of stress responses in mediating behavioural flexibility were mostly done in laboratory rodents using stressors and cognitive challenges unrelated to the ecology of the species. To better understand how stress mediates behavioural flexibility in a natural context, direct manipulations of the stress response and cognitive tests in ecologically relevant contexts are needed. To this aim, we pharmacologically blocked glucocorticoid receptors (GR) in adult *Neolamprologus pulcher* using a minimally invasive application of a GR antagonist. GR blockade prevents the recovery after a stressful event, which we predicted to impair behavioural flexibility. After the application of the GR antagonist, we repeatedly exposed fish to a predator and tested their behavioural flexibility using a detour task, i.e. fish had to find a new, longer route to the shelter when the shortest route was blocked. While the latencies to find the shelter were not different between treatments, GR blocked fish showed more failed attempts during the detour tasks than control fish. Furthermore, weak performance during the detour tasks was accompanied by an increase of fear related behaviours. This suggests that blocking GR changed the perception of fear and resulted in an impaired behavioural flexibility. Therefore, our results support a causal link between the capacity to recover from stressors and behavioural flexibility in *N. pulcher* with potential consequences for an effective and adaptive coping with changing environments.

Introduction

Stress is a ubiquitous feature of life and all organisms have evolved internal mechanisms to cope with external stressors (Taborsky et al., 2021). Stressors can be abiotic or biotic, ranging from extreme temperatures to predator attacks or social stressors such as aggressive conflicts. An adequate response to a stressor, the so-called stress response, has major fitness consequences because it is of utmost importance for the survival of the individual (Lemonnier et al., 2022; McEwen and Wingfield, 2003; Romero et al., 2009; Sopinka et al., 2015).

When a stressor is experienced the adaptive stress response in vertebrates is generally characterised by a physiological increase of circulating glucocorticoids (GCs) (Sapolsky et al., 2000). Cortisol is the major GC and a main actor of the stress response in fish and most mammals (Sopinka et al., 2015). Cortisol can cross the blood-brain barrier where it binds to high affinity mineralcorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs) that are expressed across the brain (Joels et al., 2008; Roozendaal et al., 2009). Cortisol acts on GRs and MRs to induce a negative feedback that reduces the further release of cortisol, which slowly returns to baseline levels (de Kloet, 2014; Joels et al., 2008). In contrast to mammals, most teleosts have two GRs with different hormone sensitivities (Bury, 2017). MRs also play a different role in teleosts because MR mediates the effects of aldosterone, which is absent in fish (Faught and Vijayan, 2018). The swift increase and slow decrease of cortisol is an adaptive stress response leading to beneficial changes in physiology, behaviour and cognition to successfully cope with the stressor. On the contrary, if cortisol concentrations do not return to baseline levels after the stressor ends, prolonged elevation of cortisol has adverse effects on health and behaviour particularly on cognitive abilities (Sandi and Pinelo-Nava, 2007; Schwabe et al., 2012).

The flexible adjustment of behaviours, or behavioural flexibility, requires different cognitive abilities including learning and the seeking of alternative solutions (Sol and Lefebvre, 2000; Tebbich et al., 2010). To find an alternative solution animals have to control and supress a predisposition for a

behavioural response to show a more appropriate behaviour (i.e. inhibitory control), which is a core executive function and is beneficial to animals in changing environments (Burkart et al., 2017; Diamond, 2013; Triki et al., 2023). GR activity has been shown to play an important role in influencing cognitive function and particularly in regulating learning performances (for reviews see: de Kloet et al., 2005; Roozendaal et al., 2009; Sandi and Pinelo-Nava, 2007). Previous studies using laboratory rodents have shown that GRs play a causal role in determining memory formation (Oitzl and de Kloet, 1992; Roozendaal and McGaugh, 1997), fear conditioning (Donley et al., 2005), and reversal learning, a commonly used proxy for behavioural flexibility (Bryce and Howland, 2015). Studies that tested the effects of the stress axis on behavioural flexibility in adult individuals of various species manipulated mostly type or duration of stressor (e.g. Bryce and Howland, 2015; Butts et al., 2013; Calandreau et al., 2011; George et al., 2015; Graybeal et al., 2014; Graybeal et al., 2011; Thai et al., 2013). Some studies have investigated the role of GR activitiy for learning and behavioural flexibility by manipulating GR mediated action. For example, laboratory rats receiving the specific GR antagonist mifepristone performed worse in a spatial learning task than rats receiving a control treatment (Oitzl and de Kloet, 1992). Other studies have focused on functional consequences of experiencing stressors and the resulting variation in cognition without assessing the underlying physiological mechanisms (Ferrari, 2014; Potvin, 2017; Pouca et al., 2021). To reveal that adaptive stress responses help animals to cope with changing environments we need to directly link physiological parameters involved in the recovery from stressors with the flexible adjustment of behaviour.

Fear conditioning and fear related behaviours are tightly linked with the activity of GR as well as GC concentrations (Schulkin et al., 2005a). Particularly GRs play an important role in the retention of fear memory and fear extinction after repeated exposures to a stressor (Cordero and Sandi, 1998; Kolber et al., 2008; Korte, 2001). For example, chronically stressed rats receiving a GC antagonist displayed more fear related behaviour to the conditioned stimulus alone compared to chronically stressed rats receiving a vehicle injection (Conrad et al., 2004).

102 In this work, we administered with a minimally invasive method a high dose of a GR antagonist
103 (mifepristone) to adult *Neolamprologus pulcher* and tested their behavioural flexibility in a spatial
104 task after being repeatedly exposed to a predator. Mifepristone is an antiprogestin and GR
105 antagonist with several clinical applications (Spitz, 2003). At higher dosages it mainly acts as a GR
106 antagonist (Alderman et al., 2012; Carbajal et al., 2023; Lawrence et al., 2017; Molitch, 2022; Oitzl
107 and de Kloet, 1992; Ros et al., 2012; Veillette et al., 2007) with an affinity to GRs that is tenfold
108 higher than that of cortisol (Molitch, 2022). In contrast, at lower dosages it mainly acts as an
109 antiprogestin interacting with progesterone receptors (PRs) and as such, it is also used as a non-
110 invasive method to terminate pregnancies (Blüthgen et al., 2013; Spitz et al., 1996). In the spatial
111 task, individuals had to navigate through a maze to hide in a shelter after encountering a predator.
112 After displaying flight responses using the shortest route to the shelter, we tested each individual`s
113 ability to find a new route to the shelter when the shortest route was blocked as a measure for
114 behavioural flexibility. Furthermore, as a measure for their perceived fear after being exposed to a
115 predator we recorded whether fish showed a strong fear response and hid inside the shelter or a
116 weak fear response without entering the shelter.

117 Our study species *N. pulcher* is a highly social cichlid and in nature individuals live in groups
118 consisting of dominant and subordinate individuals that are structured in a size-based, linear
119 hierarchy (Taborsky, 2016; Wong and Balshine, 2011). Groups defend territories containing crevices
120 and holes which are used as shelters for breeding and hiding from predators. Sexually mature *N.*
121 *pulcher* feed on zooplankton in the water column which requires to stay far away from their shelters
122 (Taborsky, 2016). Thus, flexibility in the way how individuals return to their shelter in case of danger
123 is of utmost importance for their survival. Organizational effects of stress in early life are well
124 researched in *N. pulcher* (Antunes et al., 2021a; Mileva et al., 2009) and pharmacologically blocked
125 GRs during early life clearly altered the development of social competence and cognitive abilities
126 (Nyman et al., 2018; Reyes-Contreras et al., 2019; Reyes-Contreras and Taborsky, 2022).

127 Nevertheless, it is not known whether a short-term application of a GR antagonist immediately
128 before a cognitive challenge mediates cognitive abilities and in particular behavioural flexibility.

129 Based on the role that GRs play in the negative feedback mechanism to terminate stress responses
130 we formulated two predictions: (1) GR antagonist-treated fish show impaired behavioural flexibility,
131 particularly after repeated exposures to a predator. (2) Effects that are mediated by GRs are specific
132 to behavioural flexibility and the ability to cope with stress in terms of fear related behaviours and
133 do not affect other behaviours such as the motivation of the fish to solve the task.

134

135 **Methods**

136 *Subjects and housing*

137 The experiment was conducted at the Konrad Lorenz Institute of Ethology, University of Veterinary
138 Medicine, Vienna, Austria, under the licence (ETK-101/06/2019). All experimental tanks (45L, 30 x 50
139 x 30 cm) were equipped with a 2 cm layer of sand, a biological filter, a heater, and one-half clay
140 flowerpot served as a shelter. The water temperature was kept at 27 ± 1°C with a light-dark regime
141 of 13:11 h and a dimmed-light phase of 10 min. During the experiment all fish were fed commercial
142 flake food 6 days a week.

143 Our study species was the cooperatively breeding cichlid *N. pulcher*, which is a model to study the
144 evolution of vertebrate sociality highlighting their social and non-social behavioural flexibility
145 (Taborsky, 2016; Wong and Balshine, 2011). These fish are endemic to Lake Tanganyika and groups
146 always consist of a dominant breeding pair and related or unrelated helpers (Balshine et al., 2001;
147 Heg et al., 2005; Taborsky, 1984, 1985). Helpers assist the breeding pair in raising the offspring by
148 cleaning and fanning the eggs, cleaning the breeding shelters, and defending the common territory
149 (Grantner and Taborsky, 1998; Taborsky, 1984, 1985). In larger territories each group member
150 occupies and defends an own shelter. As an ecologically relevant stress stimulus, we used the

151 piscivorous cichlid *Lepidiolamprologus elongatus* which occurs in sympatry with *N. pulcher* (Ochi and
152 Yanagisawa, 1998). *L. elongatus* preys on all sizes of *N. pulcher*, except eggs, and is one of its most
153 important predators (Groenewoud et al., 2016). Previous research indicates that *N. pulcher* can
154 innately recognise this predator based on visual or olfactory cues (Fischer et al., 2014; Zöttl et al.,
155 2013). In nature, groups of *N. pulcher* face a high predation pressure and individuals either attack or
156 hide when predators intrude their territories, particularly when faced with a large predator such as
157 *L. elongatus*.

158 Our test fish were laboratory-reared first- and second-generation offspring of wild-caught fish. We
159 randomly selected 35 male and 35 female adult fish (N=70, mean standard length [SL; from the tip of
160 the snout to the end of the caudal peduncle]: 7.2 cm, [range: 5.6-8.2 cm]) from the laboratory stock,
161 where they had been kept before as groups separated by sex. The experiment was conducted in
162 seven blocks over seven weeks with 10 test fish per block. Test fish were permanently marked using
163 subcutaneous injections of Visible Implant Elastomer tags (VIE; Northwest 191 Marine Technology).
164 Only fish which had been marked at least three days before the behavioural tests were included in
165 the analyses reducing the final sample size to N=60.

166 We also randomly chose adult *L. elongatus* (N = 10, mean SL: 12.68 cm [range: 10.3-14.7 cm]) from
167 the laboratory stock population, where they had been housed in pairs or small groups. We used five
168 individuals for the first three blocks and another five individuals for the last four blocks. This ensured
169 that predators behaved naturally throughout the experiment and were not exposed to excessive
170 stress due to being caught and transported to the presentation tanks repeatedly (see below). During
171 the experiment, each predator was kept in a separate 45L tank. These tanks were placed close to the
172 tanks of the test fish but with a visual and olfactory separation. Body sizes of predators were
173 obtained after the last block of the experiment was completed. All fish were kept under the same
174 housing conditions and food regimes before and during the experiment.

175

Experimental set-up

During the trials, we visually separated the experimental tanks using opaque dividers. The mazes used to carry out the detour trials remained in the experimental tanks throughout all the experimental phases (see below). The tanks were separated from the observer and the rest of the room using a dark curtain to minimize any disturbances from the surrounding area. All trials were recorded from above and from the front using two web cameras (Creative Live Cam Sync HD webcam) placed inside the curtain and connected to a computer outside of the curtain. Prior to each trial, a randomly chosen predator was transported to a small presentation tank (25 x 30 cm) filled with water and placed on a small movable rack. The front of the presentation tank – the part facing the experimental tanks – was covered with an opaque divider which could be moved with a string by the observer from outside the curtain. The divider was lifted before each trial for 60s to visually expose the test fish to the predator.

For all the trials, we used a transparent, U-shaped maze that prevented fish from using the maze as a shelter. The walls of the maze were covered with a mesh-like patterning that was fine enough to clearly see though. Halved clay flowerpots were used as shelters. The shelter could be reached using two routes: either via the direct route where fish only entered the arm of the maze that contains the shelter (‘shelter arm’, see Figure 1), or via a detour route where fish entered first the arm furthest away from the shelter (‘non-shelter arm’, see Figure 1) and had to cross to the other side in the back of the maze to enter the shelter arm. The middle arm of the maze was a dead end (Figure 1). The area in front of the maze (30 x 10 cm) that faced towards the predator was used as a starting area and could be separated from the rest of the tank with a removable opaque divider. Again, using a string the observer could remotely move the divider with minimal disturbance to the fish from outside the curtain. Biological filters and heaters were removed during the trials to ensure fish could only seek shelter inside the flowerpot.

201 *Experimental phases*

202 Each block consisted of five distinctive experimental phases: a habituation phase (day 1, day 2), a
203 pre-treatment direct phase (day 3), a treatment immersion phase (day 4, day 5), a post-treatment
204 direct phase (day 6), and a detour phase (day 6).

205 Habituation. After fish were measured (SL) to the closest mm, each fish was individually transported
206 into one of the experimental tanks. Each tank included a maze, and a shelter was placed in the
207 shelter arm. Fish were allowed to habituate to the new environment for 48h. During the habituation
208 phase we removed the opaque dividers between the experimental tanks to allow fish to visually
209 interact with their neighbours.

210 Pre-treatment direct. After the habituation phase (day 3, 10a.m.), we tested whether the fish used
211 the shortest route to the shelter after being exposed to a brief predator presentation. First, the
212 observer gently guided the fish to the starting area using a small handheld net. Second, the observer
213 lowered the divider to separate the start area from the rest of the tank. Third, the observer added
214 an olfactory predator cue (15 ml of water from a predator tank) to the starting area and lifted the
215 divider inside the predator tank to visually expose the fish to the predator for 60s. Fourth, directly
216 afterwards, the observer lifted the divider separating the start area from the rest of the tank, and
217 the fish could enter the maze and seek shelter. In this phase, the entrance of the shelter faced
218 towards the starting area. Fish successfully completed this phase if they used the direct route to
219 reach the shelter within 60s without any detours. Fish that did not use the direct route (N=8) were
220 excluded from the experiment.

221 Immersion treatment. The subjects that successfully completed the previous phase (N=52),
222 underwent a 48h period of either a mifepristone (N=26, females=14) or a control immersion
223 treatment (N=26, females=13) starting on the next day (day 4, 8a.m.). The duration of the immersion
224 treatment is well within the half-life of mifepristone (84h, see Molitch, 2022) and was chosen as a
225 compromise between a maximised exposure time and a limited confinement of the fish in a small

226 space. Half of the females and half of the males were randomly assigned to one of the two
227 treatments, in a way that ensured that the observer was blind to the treatments of the fish within
228 each block. For this a second person (SF) applied the treatments to the experimental tanks without
229 the observer (ZF) present. The treatments were applied in the form of water baths following Nyman
230 et al. (2018). In short, we dissolved mifepristone (RU 484) in dimethylsulfoxide at 50 mg ml-1, then
231 serially diluted it in 0.1 M acetic acid (1:10), phosphate-buffered saline (1:100), and finally, diluted it
232 in distilled water for an immersion concentration of 400 ng l^{-1}. Controls were prepared with diluents
233 without mifepristone. Both mifepristone and control solutions were prepared and stored in a -20C
234 freezer one week prior to the experiment. The fish were immersed in 2L of water inside 3L glass
235 containers that were placed inside the experimental tank.

236 Post-treatment direct. After the immersion treatments (day 6, 9a.m.) we repeated the previous
237 direct phase to test whether (i) the fish memorised the shortest route to the shelter, and (ii) whether
238 the immersion treatments influenced the motivation or memory of fish. Seven out of 52 fish did not
239 take the direct route in this phase. This time all fish proceeded to the detour phase because they
240 already used the direct route in the pre-treatment direct phase. This ensured that (i) all fish that
241 proceeded to the detour phase had used at least once the direct route and (ii) the immersion
242 treatments had no effect on the motivation of the fish to seek shelter.

243 Detour. For the detour trials (day 6, 10:30a.m.) we blocked the direct route to the shelter arm (Fig.
244 1) with a rectangular barrier (5 x 25 cm), made from the same material as the maze. The shelter
245 entrance was turned to face away from the starting area. This ensured that fish approached the
246 shelter from the same side irrespective of whether they entered the shelter arm via the direct or via
247 the longer route (Figure 1). We used the same procedures as described for the pre-treatment direct
248 phase. Each fish experienced 4 detour trials with a 2h recovery period between each trial.

249

250 *Video analysis*

Video recordings were analysed using BORIS (Friard and Gamba, 2016). One trial (block 3, detour 1) was excluded from the analysis due to problems with the video quality. For each trial we recorded (1) the type of the test (post-treatment direct/detour 1/detour 2/detour 3/detour 4), (2) the latency to cross the shelter arm line, (3) the latency to enter the shelter, and (4) the strength of the fear response in terms of whether the fish entered the shelter or not. In the post-treatment direct trials, we additionally (5) recorded whether the fish used the shortest route to the shelter or not. To measure behavioural flexibility, we (6) recorded the number of failed attempts during the detour trials defined as crossing the middle arm line, attempting, and trying to cross the blocked shelter arm line, or crossing the non-shelter arm line but then returning to the starting area without entering the shelter arm. These variables allowed us to analyse the role of GRs in mediating motivation (variables 2 and 3), fear related behaviours (variable 4) and behavioural flexibility (variable 6). A fish was classified as having crossed into a new arm when both pectoral fins crossed the virtual line separating the two arms. We started measuring the latencies immediately after the predator exposure – i.e. immediately after the divider in the presentation tank was closed and the divider in the experimental tank was lifted. If fish did not cross any arm line or entered the shelter before the end of the experiment, we assigned maximum latencies (600s) in the respective trial.

Statistical analysis

Data were analysed using R 4.1.1. (R Core Team, 2021) with the packages “lme4” (Bates et al., 2015) and “afex” (Singmann et al., 2021). We analysed treatment effects using Linear Models (LMs), Generalized Linear Models (GLMs), Linear Mixed Models (LMMs) and Generalized Linear Mixed Models (GLMMs). Residuals and Q/Q-plots of all LMs and LMMs were visually inspected, and the distributions of residuals were compared to a normal distribution using Kolmogorov-Smirnov and Shapiro tests. If residuals were not normally distributed, a log transformation was applied, and residuals again checked. All GLMs and GLMMs were checked for over-dispersion, but none required

276 a correction. To obtain p-values, we used either the mixed() function in the package 'afex' with a
277 Satterthwaite's approximation for degrees of freedom, or the drop1() function to perform either a
278 type II anova (for LM and LMM) or a χ^2-test (for GLM and GLMM). Interactions, and control variables
279 (sex and body size of test fish) were stepwise removed if non-significant (Engqvist, 2005) and
280 reported p-values refer to the final model without non-significant interactions, and control variables.
281 An overview of all full models can be found in Supplementary Table S1.

282 Except for the analyses of the probability to enter the shelter with increasing numbers of predator
283 exposures (see below) we always included 'Treatment' (GR antagonist or control treatment), the
284 body size and sex of test fish in the models. When analysing the detour trials, we additionally
285 included test fish identity as a random effect to control for multiple observations on the same
286 individual and the number of the detour trial as a fixed effect. If the interaction 'No. of detour trial x
287 Treatment' was significant we compared the marginal means of both treatments within each detour
288 trial separately (four comparisons) with a Tukey correction for multiple testing. To analyse the
289 probability to enter the shelter with increasing numbers of predator presentations we used all trials
290 that were done after the fish received their respective treatments (post-treatment direct, detour 1,
291 detour 2, detour 3, detour 4). Fish identity was included as a random effect and the number of daily
292 predator presentations as a continuous covariate. Initially we constructed two models, one that
293 included a linear interaction between the number of daily predator presentations and treatment and
294 one that included a non-linear, polynomial relationship with a degree of 2. We compared both
295 models using a χ^2- goodness of fit test. To foster comparability, we kept the structure of the models
296 similar and did not include the body size or sex of fish in this analysis. To further investigate the
297 significant non-linear interaction, we constructed two additional models separately for GR blocked
298 and control fish. Both models included the identity of the fish as a random factor and the number of
299 daily predator presentations as a linear predictor or as a non-linear polynomial predictor with a
300 degree of 2.

Results

Does the application of a GR antagonist influence memory or motivation to seek shelter in the post-treatment direct trials?

We found no indication that the application of a GR antagonist influence memory or motivation because the majority of fish (87%) used the shortest route to the shelter again in the post-treatment direct trials and this is significantly higher than a random expectation of 50% (binomial test, $p<0.01$). Furthermore, the immersion treatments did not influence the choice of fish to use the short or the longer route (control: 4 out of 26 fish took the longer route; mifepristone: 3 out of 26 fish took the longer route; chi-square-test, $\chi2=0.17$, $p=0.68$) or any other behaviours in the post-treatment direct trials. There were no significant differences between the two immersion treatments in the latency to cross the shelter line ($F_{1,50}=0.08$, $p=0.78$; see factor: 'Treatment' in Table 1a), and the latency to enter the shelter ($F_{1,50}=0.1$, $p=0.77$; see factor 'Treatment' in Table 1b).

Does the application of a GR antagonist influence behavioural flexibility in the detour trials during repeated exposures to a stressor?

GR blocked fish showed less behavioural flexibility during the detour trials than control fish. That is, the application of a GR antagonist influenced the number of errors that fish made over time with repeated exposures to predators ($\chi^2=23.96$, $p<0.01$, $N=52$; see interaction 'No. of detour trial x Treatment' in Table 2, Figure 2). Post-hoc analysis revealed that GR blocked fish made significantly more errors in the third detour trial compared to control fish (see Table 3 for pairwise comparisons). However, GR blocked fish did not show a longer latency to enter the shelter arm ($F_{1,50.11}=0.0$, $p=0.97$, see factor 'Treatment' in Table 4a) or a longer latency to enter the shelter ($F_{1,48.98}=0.42$, $p=0.52$, see

325 factor 'Treatment' in Table 4b) than the control fish. Overall, all fish reduced the latency to enter the
326 shelter arm with increasing predator exposures ($F_{1,152.25}$=3.78, p=0.01; see factor 'No. of detour trial'
327 in Table 4a), whereas this was not the case for the latency to enter the shelter ($F_{1,152.18}$=0.6, p=0.61;
328 see factor 'No. of detour trial' in Table 4b). Irrespective of the treatments, we found a positive
329 relationship between body size and the latency to enter the shelter ($F_{1,48.9}$=5.1, p=0.03; see factor
330 'Body size' in Table 4b) indicating that larger fish took longer to enter the shelter.

331

332 *Does the application of a GR antagonist influence fear related behaviour with repeated exposures to*
333 *a stressor?*

334 When analysing the probability of fish to enter the shelter with increasing numbers of daily predator
335 exposures, we found that a model containing a non-linear interaction between the number of daily
336 predator exposures and treatment explained the data significantly better than a linear interaction
337 term ($\chi 2$=15.37, p<0.01). GR blocked fish showed a linear reduction in the probability to enter the
338 shelter with increasing numbers of daily predator exposures (z=-2.17, p=0.03, N=62; see factor 'No.
339 of predator exposure' in Table 5a). In contrast, control fish showed a non-linear, U-shaped
340 relationship between the probability to enter the shelter and the number of daily predator
341 exposures [z=3.26, p<0.01, N=26; see factor 'poly (No. of predator exposure, 2)' in Table 5b]. Here,
342 control fish entered the shelter more frequently during the initial and the last predator exposures
343 than during any of the intermediate predator exposures (Figure 3).

344

345 **Discussion**

346 In the present experiment we applied a high dosage of a GR antagonist or a control immersion to
347 groups of fish by a minimally invasive method, and subsequently tested their behavioural flexibility
348 after repeated exposures to an ecologically relevant stressor. Fish treated with a GR antagonist (1)

349 made more errors and (2) had a higher probability to enter the shelter during the intermediate
350 compared to the initial and last predator exposures. Furthermore, the application of a GR antagonist
351 did not influence the individual motivation to participate in the task or their spatial memory. More
352 specifically, we did not find any behavioural differences during the post-treatment direct trials
353 where test fish could enter the shelter using the shorter, direct route. We introduced this phase to
354 control for potential influences of the application of a GR blocker on the motivation and memory of
355 fish.

356 In our study fish had to find a new route to avoid a predator. Thus, our results show that GRs play an
357 important role for behavioural flexibility in an ecologically relevant spatial task when repeatedly
358 exposed to a mild naturalistic stressor. Behavioural differences between GR blocked and control fish
359 were only apparent in the cognitively more demanding detour trials where inhibition control is
360 required to successfully solve the task. This matches the results of Jungwirth et al. (2024), where the
361 authors used a detour task in wild groups of *N. pulcher* and found that inhibition control was
362 affected by the difficulty of the task. Although our study is not sufficient to reveal the precise
363 underlying physiological mechanisms that drive the observed behavioural differences, the results
364 highlight a potential role of fear in mediating behavioural flexibility: we found that the differences
365 between GR blocked and control fish in terms of fear related behaviour (entering the shelter after
366 predator exposure) and behavioural flexibility (number of failed attempts in the detour trials)
367 followed the same pattern. In both behaviours, the difference between GR blocked and control fish
368 was greatest during the second and third detour trial and both the number of failed attempts and
369 the probability to enter the shelter were higher in GR blocked fish than in controls. GRs are
370 distributed across the entire brain but GR activity in the basolateral amygdala and the ventral
371 hippocampus have been specifically linked to the retention of fear memory (Donley et al., 2005).

372 Possibly, the difference in fear related behaviour was associated with increased concentrations of
373 circulating GCs, in our case cortisol because previous studies showed that GCs are essential for fear

374 related behaviours (for review see Schulkin et al., 2005b). Cortisol might have increased more in GR
375 blocked fish due to their reduced capacity to terminate the stress response before encountering the
376 next stressful predator exposure. However, another study in rainbow trout found a reduction in GC
377 concentrations in GR blocked fish after exposure to a single stressor (Alderman et al., 2012).
378 Nevertheless, it remains to be determined how GR activity influences GC concentrations after
379 repeated exposures to the same stressor.

380 Although we did not directly measure cortisol, we observed a clear difference in fear related
381 behaviours between the GR blocked and control fish. The response of control fish followed a U-
382 shaped function with more fear related behaviour during the first and last predator presentations
383 than during the intermediate presentations. The response of GR blocked fish followed a linear
384 function with a continuous reduction of fear related behaviours from the first to the last predator
385 presentation. We can think of three non-mutually exclusive explanations for this result. (1) In control
386 fish the initial faster decline in fear related behaviour might reflect a fast habituation to the
387 exposure to the stressor. In contrast, if GR blocked fish had increased cortisol levels due to the
388 disruption of the negative feedback mechanism, they might have experienced chronic stress earlier
389 which resulted in a slower habituation to the repeated stress exposure (Cyr and Romero, 2009). (2)
390 Alternatively, the faster decline might indicate that control fish were better able to cope with the
391 repeated exposure to stressors. A faster stress recovery may alter the potential responsiveness to a
392 future stressor or aid in adapting to a chronic stressor (Sapolsky et al., 2000) (3) Finally, the
393 subsequent increase of fear related behaviour in control fish might be explained by a potential
394 ceiling effect for the facilitating action of stress during the acquisition of spatial information. Similar
395 to our results, rats injected with an intermediate dosage of GCs performed better in a spatial
396 learning task than rats injected with a low or a high dosage (Sandi and Pinelo-Nava, 2007). Further
397 investigations are needed to disentangle the complex interplay between GCs, GRs and cognition
398 particularly when repeatedly experiencing a stressor during development or later in life.

399 Mifepristone has several clinical applications and at low dosages it mainly acts as an antiprogestine
400 whereas at higher dosages it mainly acts as a GR antagonist (Spitz, 2003). Our immersion treatment
401 allowed us to apply a high dosage (400 ng l^{-1}) of mifepristone which is more than fivefold than the
402 highest dosage used in another study to investigate the effects of mifepristone as an antiprogestin in
403 fish (Blüthgen et al., 2013). Thus, in our experiment, mifepristone acted mainly as a GR antagonist
404 rather than as an antiprogestine, which limits the possibility that our results are explained by
405 potential interactions between mifepristone and PRs. In line with this conclusion, we did not detect
406 any sex specific effects on the observed behaviours. Nevertheless, progesterone is known to
407 determine cognitive functions, particularly in humans (Griksiene et al., 2022) and future studies
408 investigating the potential dose-dependent effects of mifepristone on cognitive abilities in *N. pulcher*
409 are needed.

410 As in many animals, in *N. pulcher*, social and ecological experiences during early life are of particular
411 relevance for the development of social and anti-predator behaviours, as well as for non-social
412 behavioural flexibility (Bannier et al., 2017; Fischer et al., 2017). These organisational effects are
413 most likely mediated by a life-long reprogramming of the physiological stress axis (Antunes et al.,
414 2021a; Antunes et al., 2021b; Nyman et al., 2018) although an early-life manipulation of the stress
415 axis using the same GR antagonist did not affect behavioural flexibility when adult individuals were
416 tested in a reversal learning paradigm (Reyes-Contreras and Taborsky, 2022). In contrast, our study
417 shows an activational effect on behavioural flexibility mediated by differences in the immediate
418 stress response to a predator. Future studies focusing on both the organizational and the
419 activational effects of the stress response on cognition, in particular on behavioural flexibility are
420 needed to better understand the way how animals respond to naturalistic environmental challenges.

421 In conclusion, the current study provides novel insights into the mechanisms of adapting to
422 environmental change. We used an ecologically relevant stressor and cognitive task to highlight the
423 role of GRs in regulating fearful behaviour and mediating behavioural flexibility. We encourage

further research to disentangle the interactions between stress responsiveness, cognitive abilities and associated behaviours in a natural context.

Data availability

Data for this study are available at Figshare public repository:
https://doi.org/10.6084/m9.figshare.24083952

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Author Contributions

Conceptualization & funding acquisition: K.H., B.T., L.F., S.T.; Data curation: S.F; Formal analysis: S.F., Investigation: S.F., Z.F.; Methodology: S.F., Z.F. S.T., Project administration: S.F., L.F., S.T.; Supervision: S.F., S.T.; Visualization: S.F.; Writing-original draft: S.F., Z.F.; Writing-review & editing: all authors (categories based on Contributor Role)

Declaration of Interest

We declare no conflict of interest.

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Factors	Estimate ± SE	Num. D.F.	Den. D.F.	F-value	*P*-value
(a) Latency to enter the shelter arm					
Intercept	2.92 ± 0.31	-	-	-	-
Treatment	0.12 ± 0.44	1	50	0.08	0.78
(b) Latency to enter the shelter					
Intercept	3.29 ± 0.3	-	-	-	-
Treatment	0.12 ± 0.42	1	50	0.1	0.77

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733 **Table 1**: Latencies recorded during the post-treatment direct trials. (a) The latency to enter the
734 shelter arm, and (b) the latency to enter the shelter were not significantly different in glucocorticoid
735 receptor antagonist treated or control treated fish. The dependent variables were log transformed
736 to obtain normally distributed residuals. Estimates are shown on a log scale and as differences to the
737 reference level 'control' for factor 'Treatment'. Body size and sex of test fish (N=52) did not predict
738 any of the latencies and were subsequently removed from the models. F-values and p-values were
739 obtained using likelihood ratio tests comparing models with or without the factor of interest.

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Factors	Estimate ± SE	X^2-value	*P*-value
Intercept	1.44 ± 0.18	-	-
Treatment	-0.26 ± 0.26	0.65	0.42
No. of detour trial	-*	288.56	**<0.01**
No. of detour trial x Treatment	-*	23.96	**<0.01**

745 *Estimates are shown in Table 4

746

747 **Table 2**: Number of failed attempts during the four detour trials. Fish treated with a glucocorticoid
748 receptor (GR) antagonist showed a significantly different learning curve during the detour trials
749 compared to control treated fish. (see significant interaction 'No. of detour trials x Treatment'). Post-
750 hoc tests revealed that GR blocked fish showed significantly more errors during detour trial 3 than
751 control fish (see Table 3 for the pairwise comparisons). Estimates are shown on a log scale and as
752 differences to the reference level 'control' for factor 'Treatment'. Estimates for the factor 'No. of
753 detour trials' and for the interaction 'No. of detour trials x Treatment' are presented as post-hoc
754 comparisons in Table 3. Body size and sex of test fish did not predict the number of a failed attempts
755 and were subsequently removed from the model. X^2 and p-values were obtained from likelihood
756 ratio tests comparing models with or without the factor of interest. N=52 (207 observations in total).
757 P-values < 0.05 are highlighted in bold.

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Contrast	Estimate ± SE	z-value	*P*-value
Detour 1			
Control vs Mifepristone	0.26 ± 0.26	1	0.31
Detour 2			
Control vs. Mifepristone	-0.62 ± 0.37	-1.7	0.09
Detour 3			
Control vs. Mifepristone	-1.15 ± 0.42	-2.72	**0.01**
Detour 4			
Control vs. Mifepristone	0.64 ± 0.41	1.57	0.12

764

765 **Table 3:** Post-hoc comparisons with a Tukey correction of the significant interaction term in Table 2.
766 Fish treated with a glucocorticoid receptor antagonist made significantly more errors during detour
767 trail 3 leading to a significantly different error pattern in the detour trials (see Figure 2). Estimates
768 are shown on a log scale, the direction of comparison within a contrast is left to right and the
769 estimate values are shown as difference to the level at the left. N=52 (207 observations in total). P-
770 values < 0.05 are highlighted in bold.

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Factors	Estimate ± SE	Num. D.F.	Den. D.F.	F-value	*P*-value
(a) Latency to enter the shelter arm					
Intercept	4.92 ± 0.19	-	-	-	-
Treatment	0.01 ± 0.23	1	50.11	0.0	0.97
No. of detour trial	-	3	152.25	3.78	**0.01**
Detour 2	-0.49 ± 0.16	-	-	-	-
Detour 3	-0.39 ± 0.16	-	-	-	-
Detour 4	-0.36 ± 0.16	-	-	-	-
(b) Latency to enter the shelter					
Intercept	1.71 ± 1.6	-	-	-	-
Treatment	-0.14 ± 0.22	1	48.98	0.42	0.52
Body size	0.5 ± 0.22	3	48.9	5.1	**0.03**
No. of detour trial	-		152.18	0.6	0.61
Detour 2	-0.2 ± 0.2	-	-	-	-
Detour 3	-0.19 ± 0.17	-	-	-	-
Detour 4	-0.18 ± 0.17	-	-	-	-

775

776 **Table 4:** Latencies recorded during the detour trials. (a) The latency to enter the shelter arm, and (b)
777 the latency to enter the shelter was not significantly different in glucocorticoid receptor antagonist
778 treated or control treated fish. The dependent variables were log transformed to obtain normally
779 distributed residuals. Estimates are shown on a log scale and as differences to the reference levels
780 'control' for factor 'Treatment' and 'Detour 1' for factor 'No. of detour trial'. Sex of test fish did not
781 predict the latencies and was removed from the models, whereas body size was only dropped in (a).
782 F-values and p-values were obtained from likelihood ratio tests comparing models with or without
783 the factor of interest. N=52 (207 observation in total). P-values < 0.05 are highlighted in bold

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Factors	Estimate ± SE	z-value	*P*-value
(a) GR blocked fish			
Intercept	2.07 ± 0.47	-	-
No. of predator exposure	-7.69 ± 3.54	-2.17	**0.03**
poly(No. of predator exposure, 2)	3.42 ± 3.18	1.08	0.28
(b) Control fish			
Intercept	1.8 ± 0.51		
No. of predator exposure	-4.23 ± 3.5	-1.21	0.23
poly(Nr. of predator exposure, 2)	11.87 ± 3.64	3.26	**<0.01**

788

789 **Table 5:** The probability to enter the shelter with increasing numbers of daily predator
790 presentations. Fish treated with a glucocorticoid receptor (GR) antagonist showed a significantly
791 different pattern over the course of the experiment than control treated test fish. Separate models
792 were performed only after confirming that a non-linear interaction term between the number of
793 daily predator presentations and treatment explained the data better than a linear relationship (see
794 Results section). (a) GR blocked fish show a linear decrease in the probability to enter the shelter
795 with increasing daily predator presentations, whereas (b) control fish show a significant U-shaped
796 relationship (see also Figure 3). Estimates are shown on a logit scale. The factor 'No. of predator
797 exposure' was included as a continuous variable either as a linear predictor or as a polynomial
798 predictor (poly) of degree 2. N=26 (a: 129 observations; b: 130 observations in total).

799

800

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802

803 **Figure legends**

804 **Figure 1:** Experimental set-up consisting of one predator tank and a 45L experimental tank. The
805 predator tank contained a removable opaque divider that could be remotely lifted during the
806 predator presentations. The experimental tank was divided into five areas that are indicated with
807 letters (a)-(e): (a) The start area which could be separated by a removable opaque divider from the
808 rest of the experimental tank. (b) The non-shelter arm which served as the correct entrance in the
809 detour trials. (c) The middle arm leading to a dead end. (d) The shelter arm that always contained a
810 half clay flowerpot with the opening either facing towards the start area during the direct trials or
811 with the opening facing away from the start area during the detour trials (as depicted here). (e) The
812 back side arm which test fish had to use to enter the shelter arm in the detour trials. The U-shaped
813 maze consisted of transparent Perspex material. A mesh was glued on top to make the maze clearly
814 recognizable. Dashed lines indicate virtual lines that were used to record latencies and failed
815 attempts from the video recordings (see Methods section for more details). The shortest route
816 towards the shelter was either directly accessible (direct trials) or blocked (detour trials) with a
817 barrier made from the same material as the maze.

818

819 **Figure 2:** Behavioural flexibility as measured by the number of failed attempts in the detour trials.
820 Glucocorticoid receptor (GR) antagonist treated fish showed a different pattern of failed attempts in
821 the detour trials than control treated fish. GR blocked fish made more errors particularly during
822 detour trial three. Individual data points (smaller circles and triangles in the background) and
823 predicted values (larger circles and triangles in the front) are shown with 95% confidence intervals
824 for control (red circles and solid line) and GR blocked (blue triangles and dashed line) fish.

825

826

827 **Figure 3:** Fear-related behaviour measured by the probability to enter the shelter with increasing
828 numbers of daily predator presentations for control treated (red circles and solid line) and
829 glucocorticoid receptor (GR) antagonist treated (blue triangles and dashed line) fish. GR blocked fish
830 showed a constant decrease in the probability to enter the shelter with successive predator
831 presentations whereas control fish showed a nonlinear U-shape relationship. Circles and triangles
832 represent raw data and lines represent model predictions with standard errors depicted as shaded
833 areas.

834

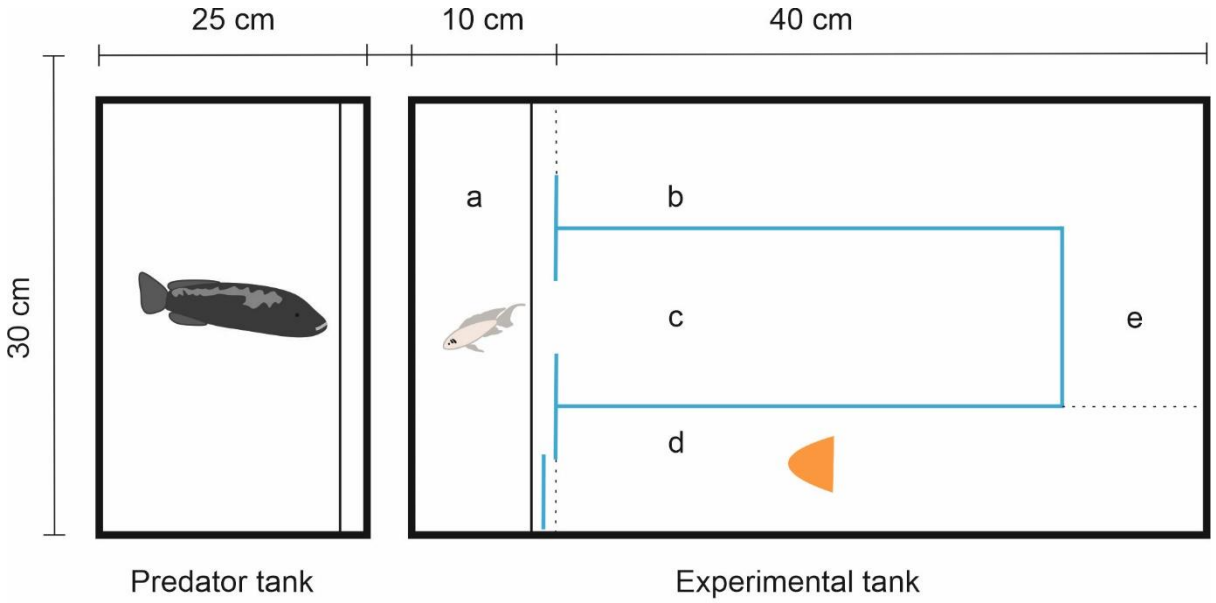
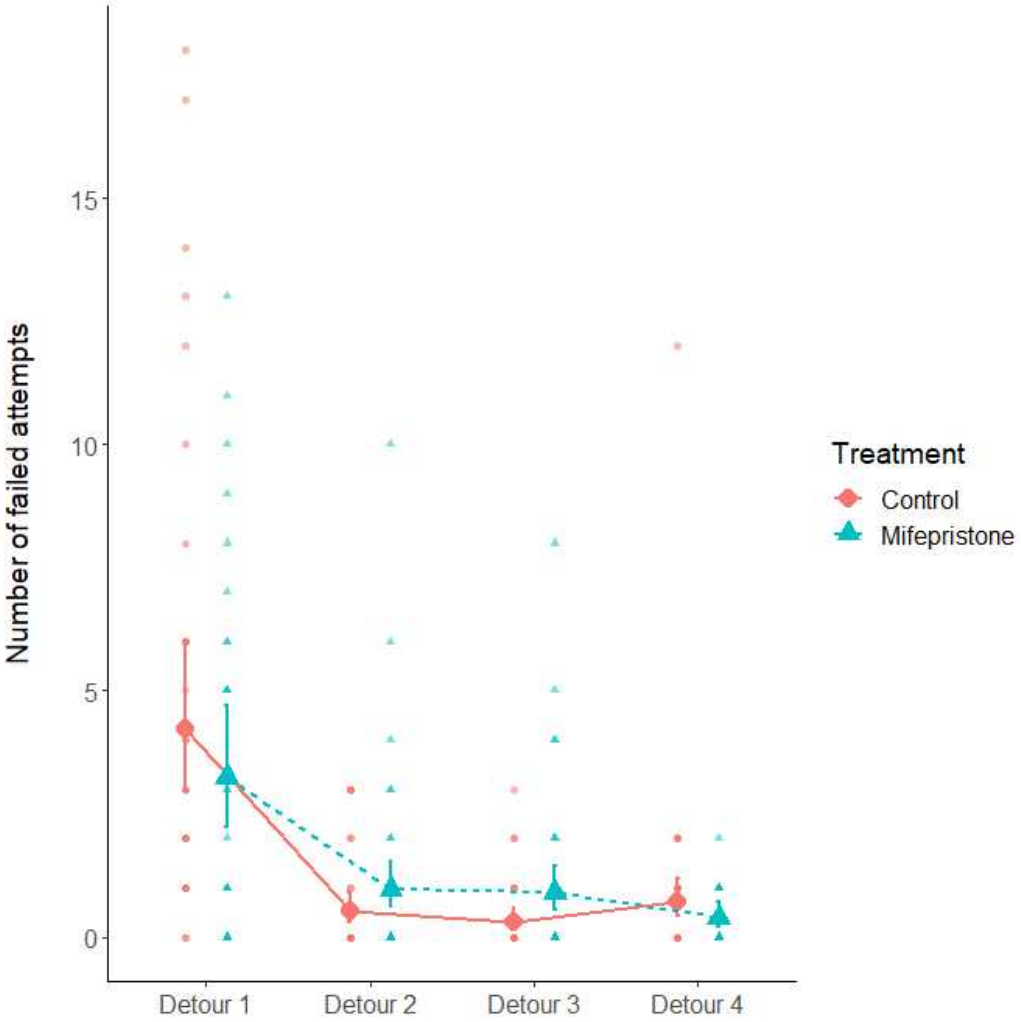


Figure 1

840

841

842 Figure 2



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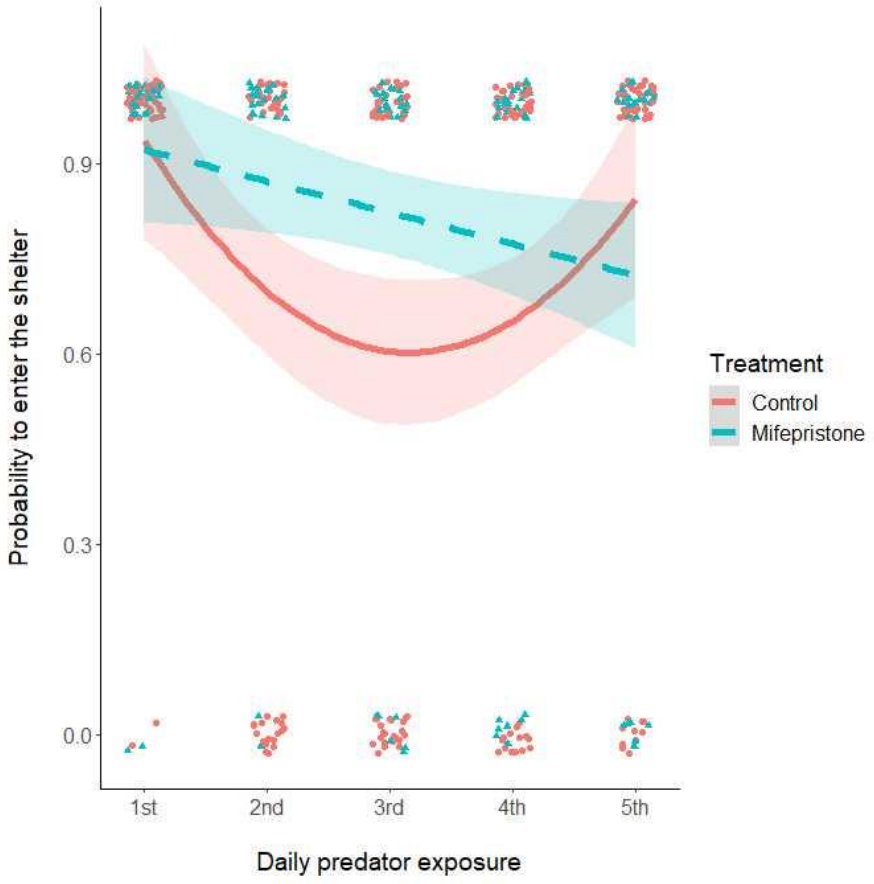
845

Figure 3


Probability to enter the shelter
0.9
0.6
0.3
0.0
1st
2nd
3rd
4th
5th
Daily predator exposure
Treatment
Control
Mifepristone