Nutritional state and predator exposure jointly mediate stress resilience and behavioural flexibility

Stefan Fischer[1,2], Katharina Hirschenhauser[3], Barbara Taborsky[4], Leonida Fusani[1,2], Virginie Canoine[2] & Sabine Tebbich[2]

[1]Konrad Lorenz Institute of Ethology, Department of Interdisciplinary Life Sciences, University of Veterinary Medicine Vienna, Savoyenstrasse 1, 1160 Vienna, Austria

[2]Department of Behavioral and Cognitive Biology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria

[3]University College for Education of Upper Austria (PH OÖ), Kaplanhofstraße 40, 4020 Linz, Austria

[4]Division of Behavioural Ecology, University of Bern, Wohlenstrasse 50a, CH-3032 Hinterkappelen, Switzerland

Correspondence and requests for materials should be addressed to S.F. (email:Stefan.fischer@vetmeduni.ac.at)

ORCIDs:

0000-0001-8811-7518 (Stefan Fischer)

0000-0001-8710-9022 (Katharina Hirschenhauser)

0000-0003-1690-8155 (Barbara Taborsky)

0000-0001-8900-796X (Leonida Fusani)

0000-0003-2272-0634 (Virginie Canoine)

0000-0003-3971-2139 (Sabine Tebbich)

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Abstract

Organisms must adapt to changing environments, and behavioural flexibility plays an important role in adaptation within an individual's lifetime. Stress resilience influences flexibility, as short-term stress can enhance attention and memory, while prolonged stress may impair cognitive function. Nutritional state also plays a role, with overweight individuals often suffering greater consequences of chronic stress. The allostatic load model predicts that these individuals, under prolonged stress, remain in a chronically activated state, potentially reducing flexibility.

Using a social cichlid, we manipulated body condition and exposed individuals to occasional or frequent predator presentations. We then assessed stress responses and behavioural flexibility via a reversal learning test. Contrary to predictions, fish in a high nutritional state exposed to frequent predators recovered rapidly from stress and exhibited enhanced flexibility. Our results provide direct evidence that nutritional state interacts with behavioural flexibility, revealing the costs of coping with repeated stressors.

We highlight a link between brief stress responses, characterized by rapid cortisol recovery, and improved flexibility. This association only emerged in well-nourished individuals facing frequent stressors, suggesting energy reserves buffer the costs of repeated stress. Thus, resilience benefits behavioural flexibility, but it is also energetically costly, as a reduced diet hindered adaptation to environmental change.

Introduction

Animals have to constantly cope with and adapt to stressors in the environment such as the presence of predators or changing environmental conditions [1]. Stress resilience, i.e. how rapid, efficient, and complete the recovery of the stress response after a perturbation has occurred [sensu 2], is a major component of behavioural and physiological adaptations for coping with extreme conditions. The hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis plays a central role in mediating physiological stress resilience [3]. This hormonal cascade induces the release of glucocorticoids (GCs), such as cortisol or corticosterone, which help mobilising energy to cope with the stressor [4]. Stressors disrupt the homeostasis of an organism, and the concept of allostasis refers to the process by which the body reestablishes homeostasis through physiological or behavioural changes [5-7]. Allostasis is costly and organisms accumulate allostatic load when exposed to stressors [8, 9]. An effective termination of the stress response is therefore essential to prevent the detrimental effects of prolonged exposure to stress hormones such as GCs [5]. Thus, acute stress responses are characterised by a short activation of the HPA/HPI axis with the result of increased GC secretion from the adrenal, followed by a swift decrease. If the stressors occur repeatedly, but only occasionally, there is sufficient time for the organism to quickly return to homeostasis, thus keeping the cost of allostasis low. An acute stress response is beneficial, because it effectively activates physiological and behavioural processes to cope with the stressor. Such acute stress responses are adaptive and important for survival with positive effects on health and cognition [10-12].

In contrast, prolonged stress responses are characterised by a long activation of the HPA/HPI axis with persistently elevated GC concentrations [13]. This persistent accumulation of allostatic load is energetically costly and may lead to 'wear and tear' of the organism's body [8]. If stressors occur frequently and the organisms cannot recover, these wear and tear costs accumulate and may contribute to a range of health problems and impair cognition [14].

Behavioural flexibility is the capacity to adapt behaviour in response to changing environmental conditions [15-17]. Arguably, cognition is among the most important determinants of behavioural flexibility because learned behaviour can be reversed, allowing for significant adaptability [18]. While exposure to acute stressors may enhance behavioural flexibility [19-21], prolonged or frequent stressors impair behavioural flexibility, possibly as a result of persistently elevated stress responses [22].

The type and the duration of the stressor as well as the energetic reserves of the organism determine whether a stressor elicits an acute or prolonged stress response [5, 6, 23]. Previous studies proposed

84 that overnutrition results in the accumulation of excessive baseline energy reserves that fuel
85 prolonged allostasis; therefore the assumption was that obese organisms would be more susceptible
86 to the effects of prolonged stress responses than lean organisms [5, 6]. The consequences can be
87 severe. For instance, in humans chronic stress has been associated to a heightened risk for obesity,
88 which in turn results in negative health consequences such as depression and an increased risk of
89 inflammations [24-26].

90 A resilient organism is able to quickly overcome the allostatic load induced by a stressor and returns
91 to homeostasis. Resilience is affected by several factors including the energetic reserves and life-
92 history characteristics of the species [2]. In humans, resilient stress response patterns reveal
93 important health considerations, e. g. poor stress recovery correlates with poor cardiovascular status
94 [27] and obesity has been linked to lower stress responsiveness and impaired cognition [28, 29].
95 Characterising the specific factors that determine resilience in humans is a medically important
96 question. However, identifying these important and diverse factors is extremely difficult, particularly
97 in humans, due to the limits of experimental manipulations [30]. Thus, more research about the
98 interaction between nutrition, stress responses and cognition is needed, particularly in animal
99 models. So far, studies in laboratory rodents and birds showed rather mixed results [31-34] with
100 positive, negative, or no relationships between body condition and glucocorticoid responsiveness. We
101 are not aware of any previous studies that have experimentally investigated the influence of
102 nutritional state on the recovery pattern of the stress response and the resulting consequences for
103 behavioural flexibility

104 Fish and in particular cichlids are excellent models to further develop the research fields of cognition
105 and stress. The physiological mechanisms to cope with stressors are evolutionary conserved among
106 vertebrates [35] and cichlids display a broad repertoire of socio-cognitive abilities [36] including
107 behavioural flexibility [37, 38]. Standard procedures for experimental stress exposure are available
108 and individuals can be easily tagged to obtain individual characteristics (e.g. body condition) [39, 40].
109 Combined with detailed physiological measurements, this allows using both functional and
110 mechanistic approaches in experiments with cichlids. *Neolamprologus pulcher* is a highly social
111 cichlid, in which numerous previous studies have demonstrated the significance of the stress axis and
112 stress reactivity for its social behaviour and cognition [35, 38, 41-44]. Thus, these fish are a well
113 suited model to test how the frequency of stressors and nutritional status influence stress response
114 patterns and behavioural flexibility.

115 Here we performed an experiment using *N. pulcher* as study organisms to test the following
116 hypothesis (1) The frequency of stressors and the nutritional state changes individual cortisol
117 response patterns which ultimately influences behavioural flexibility. To test this prediction, we

manipulated the nutritional status of individuals while exposing them to different frequencies of recurrent stressors in the form of occasional or frequent predator presentations. At the end of the experiment, we assessed individual stress response patterns to a novel stressor and behavioural flexibility using a reversal learning test paradigm [37]. Based on the allostatic load model we formulated two specific predictions: (1) Frequent exposure to stressors and overnutrition separately induce prolonged allostatic states including persistently prolonged high cortisol concentrations and impaired behavioural flexibility, whereas an occasional exposure to stressors and a lower nutritional status induce brief allostatic states. (2) When these two factors are combined, overweight individuals are particularly susceptible to the negative effects of frequent exposure to stress, compared to lean individuals.

Methods

Subjects and housing

The experiment was conducted at the Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria, under the licence (ETK-101/06/2019). All experimental tanks were equipped with a 2 cm layer of sand, a biological filter, and a heater. The water temperature was kept at 27 ± 1°C with a light-dark regime of 13:11 h and a dimmed-light phase of 10 min.

The study species was the cooperatively breeding *N. pulcher*, which is endemic to Lake Tanganyika. It lives in stable social groups always including a dominant breeding pair and related or unrelated helpers [45-48]. As an ecologically relevant stress stimulus, we used the piscivorous cichlid *Lepidiolamprologus elongatus*, which coexists with *N. pulcher* [49]. *L. elongatus* preys on *N. pulcher* of all sizes except for the eggs and is one of its most significant predators [50].

Experimental design

For this experiment we used 126 (63 male and 63 female) adult *N. pulcher* randomly selected from the institute`s stock population. The experiment was run in three separate blocks with 42 (21 females) fish each that were housed in sex separated groups of 3-7 individuals (see below and Supplementary Fig. S1). A block design with three blocks was chosen for logistical reasons and we controlled for potential temporal effects by always including all six treatments in each experimental block. To test the predictions outlined in the introduction, 30 groups of fish were created in total and each group received one of the following six treatments, resulting in five groups and a total of 21 test fish per treatment (Fig. 1 and Supplementary Fig. S2): (1) increased diet, no predators (IN); (2)

150 increased diet, occasional predators [IO]; (3) increased diet, frequent predators [IF]; (4) reduced diet,
151 no predators [RN]; (5) reduced diet, occasional predators [RO]; and (6) reduced diet, frequent
152 predators [RF]. At the end of the treatments, we randomly selected 96 test fish from each group and
153 assessed either their stress response patterns to a novel stressor (1 or 2 per group, Fig. S1) or their
154 behavioural flexibility (1, 2 or 4 per group, Fig. S1). From the remaining fish (N=30, 1 per group) we
155 collected the entire brains for a separate study that will be presented elsewhere. The experiment
156 consisted of four phases: (1) the feeding regime, (2) predator presentations, (3) reversal learning task,
157 and (4) hormone sampling. Each block of four phases lasted nine weeks (Fig. 1). Throughout the
158 experiment we constantly monitored for signs of aggression between group members, and in cases
159 where overt aggression was observed, we placed a transparent divider with holes in the tank for the
160 remainder of the experiment to physically separate fish from other group members without
161 interfering with visual or olfactory contact.

162

163 Experimental setup

164 Prior to the start of the experiment, we measured the standard length of the test fish (from the tip of
165 the snout to the end of the caudal peduncle [SL], to the nearest 0.1 cm) and body mass (BM, to the
166 nearest 0.1 g). To obtain individual growth rates (see Supplementary Figs. S6, S7) and to avoid testing
167 the same fish repeatedly, we used visible implant elastomer (VIE, Northwest Marine Technology, Inc.)
168 tags to individually mark the fish [40]. In block 1, all test fish were divided into six groups of seven,
169 separated by sex (three female and three male groups) and placed in 160L tanks. In blocks 2 and 3,
170 test fish showed more signs of aggression prior to the start of the experiment, and we decided, for
171 ethical reasons, to separate the fish into 12 groups of three (six male and six female groups) and 12
172 groups of four (six male and six female groups) again separated by sex (see Supplementary Fig. S1).

173

174 Feeding regime

175 Each group was assigned to one of two feeding regimes: (1) an increased (2) or a reduced diet. Fish
176 were fed with commercial JBL Stick M for cichlids® once a day six times per week. The increased and
177 reduced diet consisted of either 1.5 or 0.5 sticks per fish respectively (please see Supplementary
178 Methods S1 for results of a pilot experiment which confirms that the two diets induced fast and long-
179 lasting changes in body condition). We chose JBL Stick M for cichlids® as food because of two
180 reasons: (1) Individual sticks within a package are uniform and have nearly the same weight (0.045 ±
181 0.002g [mean ± SEM], N=10), which enabled us to create the two different food regimes by varying

the number of sticks provided to the groups. (2) The sticks float on the water surface for a prolonged time and this assured that all group members received an equal amount of food.

In each block the experiment started with the assignment of each group to its respective feeding regime and fish were fed the diet until the start of the hormone sampling phase (phase 4). During feeding we always placed two transparent dividers inside the tanks to assure that all fish within the same group received similar amounts of food. The dividers were arranged in a way to split up larger groups of fish into individual fish or small groups of 2-3 individuals depending on the initial group size. Particular attention was paid to avoid the repeated formation of small feeding groups that consisted of the same individuals. Dividers were left in place until most of the food was consumed and removed thereafter.

Predator presentations

After fish had received their respective dietary treatments for three weeks, each group was assigned to one of three predator presentations. In blocks 2 and 3, two groups were assigned to the same predator presentations resulting in the same number of fish per treatment overall. All predator presentations consisted of a 5 min video playback of either (1) a recording of an empty tank, without any predators visible, or (2) a recording of an attacking predator. To manipulate the frequency of predator exposure, we presented the recording of the attacking predator either three times per week (occasional exposure) or three times per day (frequent exposure).

We presented the video playbacks using a 25.7cm [10.1] display using a laptop (Lenovo Ideapad D330 81MD-0006 Tablet Top). Videos were pre-recorded and validated to elicit similar responses as live predators [39]. When presenting a video of an attacking predator, we randomly selected one of the four available videos and randomly placed the screen either left or right next to the experimental tank. To ensure that fish perceive the predators also using olfactory cues, we transferred 20ml of predator water to create a more naturalistic experience of an approaching predator. Predator water was always derived from one of two tanks (400L) containing five adult *L. elongatus* each. To avoid that test fish habituated to the predator presentations we spread out the presentations semi-randomly throughout the entire day and extended it with additional standardized stress manipulations (see Supplementary methods S2). To control for potential disturbances caused by adding predator water during the presentation of predator recordings, we also added 20ml of water from tanks containing groups of *N. pulcher* not used in the experiment during the presentation of control recordings.

Reversal learning task

We used a well-established reversal learning task to measure behavioural flexibility [37, 38]. For this, fish first had to learn a visual discrimination task and subsequently perform the reverse of the previously learned reward contingency. From each treatment group, we randomly selected 10 fish to be tested individually (in total N=60 test fish [Block 1: N=22; Block 2,3: N=18]). The task was separated in three phases: (1) pre-training and habituation phase, (2) training and colour discrimination phase and (3) reversal learning phase. One fish died before the start of the training phase reducing the overall sample size to N=59.

(1) Pre-training and habituation phase: During the last two weeks of the predator presentations, we conducted the pre-training phase to habituate the fish to the test apparatus. The test apparatus consisted of an experimental dark grey slate placed on the sandy bottom of the tank. The slate contained 16 wells arranged in 4 columns, which could be covered with coloured discs of 15 mm diameter. During the normal feeding schedules (as described above), when the groups were separated into smaller feeding groups, we also placed an experimental slate in each separation. We hid a small amount of food (crushed JBL Stick M for cichlids, according to their dietary treatments) in some of the wells which were either uncovered or only partially covered with a coloured disc (irrespective of the colour). During the two-week period we consecutively increased the coverage of the wells by the coloured discs (25%, 50%, 75%, 100%) [see 51].

(2) Training phase: After the two-week pre-training and habituation phase we randomly selected fish from each group for the reversal learning task. Each fish was weighed and measured as described above and transported to an experimental training tank (30 x 30 x 50 cm). Each training tank was equipped with a 2cm layer of sand, a biological filter, a heater, and a half clay flowerpot as shelter. Each training tank was divided into a home compartment (2/3 of the tank) and an experimental compartment (1/3 of the tank). The two compartments were separated by an opaque barrier attached to strings on pulleys, allowing the observer to lift the barrier remotely while hiding behind a curtain to minimize disturbance of the fish during the procedure. The training tanks were placed side by side to allow the fish to interact socially, ensuring the welfare of the experimental fish and encouraging their participation during the tests. During tests we placed opaque barriers between the training tanks which avoided that fish in neighbouring tanks influenced or eavesdropped on the behaviour of the focal fish. Each fish was allowed to acclimate overnight to the new environment before the training phase started. Human observers were hidden behind a curtain and observations were done blind to the experimental treatments. All observations were recorded using a web cam placed on a tripod in front of the curtain which was connected via a USB cable to a laptop at the

248 other side of the curtain. This set-up allowed us to record and directly observe each test without the
249 need to lift the curtain and potentially disturb the fish.

250 As soon as the fish had habituated to the novel experimental tank, we started to train them to access
251 food from the feeding slate. We placed a feeding slate in the experimental compartment, removed
252 the barrier to the home compartment and allowed the fish to access the experimental compartment
253 and the food on the feeding slate. In the first training trial, a small green and a blue plastic disc were
254 placed again next to one of the holes containing a reward (a red mosquito larvae). On each successive
255 trial, the discs were placed so that they covered more and more of the holes, until the discs
256 completely covered the holes with the rewards, and the fish had to learn to move the disc by pushing
257 it away to reach the reward. We always performed six trials per day for a maximum of 4 days or until
258 fish successfully moved a disc to reach a reward that was entirely covered. Once they successfully
259 moved a disc (N=56) or repeatedly entered the experimental compartment to eat the reward (N=2),
260 fish proceeded to the acquisition phase on the next day. One fish did not proceed to the acquisition
261 phase because it never entered the experimental compartment to eat the reward from the slate
262 during the habituation phase.

263 *(3) Colour discrimination in the acquisition phase*: In this phase, fish (N=58) were given the choice
264 between two coloured discs (blue and green), one with an accessible reward and one with no reward
265 underneath. To prevent fish from uncovering the non-rewarded hole, we attached a pin below the
266 unrewarded disc, which made it hard for the test fish to push the disc away and uncover the well. We
267 placed a few drops of water which had previously contained mosquito larvae but not the actual
268 larvae, into the hole covered by the unrewarded disc. This ensured that fish used visual cues (i.e. the
269 colour of the discs) and not any other cues (i.e. the smell of the reward) to discriminate between the
270 rewarded and unrewarded discs. The rewarded colour was randomly assigned so that half of the fish
271 always found the reward underneath the blue disc, while the other half of the fish found the reward
272 underneath the green disc. We always placed one disc on the left and the other disc on the right side
273 of the slate on a randomly chosen well. On the next trial, the disc previously placed on the right side
274 was placed on the left side on a randomly selected well and vice versa, and we continued to switch
275 sides on each subsequent trial.

276 After the feeding slate was prepared, the observer moved behind the curtain and lifted the barrier
277 between the experimental and the home compartment using a string. The angle of the video camera
278 was positioned in a way that allowed us to test 3 fish at the same time. After the barrier was lifted,
279 each fish was allowed to explore the experimental compartment for a maximum of 3 minutes. In a
280 successful trial, the fish first uncovered the rewarded disc and accessed the reward. Immediately
281 afterwards the observer closed the barrier and the fish moved back into the home compartment

282 waiting for the next trial to start. In an unsuccessful trial, the fish either first touched the unrewarded
283 disc or did not touch any discs until the end of the trial. If the fish first touched the unrewarded disc
284 or did not touch any discs, the observer did not close the barrier allowing the fish more time to move
285 the rewarded disc and to uncover the reward. If the fish did not move the correct disc within an
286 additional 2-minutes, the observer closed the barrier and moved the fish back into the home
287 compartment. Afterwards, the observer moved the rewarded disc to reveal the reward underneath
288 and opened the barrier until the fish consumed the reward or for a maximum of 2 min. If the fish did
289 not consume the reward within these additional 2 minutes, the observer closed the barrier again and
290 the fish moved back into the home compartment. The uneaten reward was transferred from the
291 feeding slate into the home compartment and the fish could consume the reward while the
292 preparations for the next trial took place. This ensured that all fish consumed the same amount of
293 food during each testing day. This procedure was repeated for six consecutive trials per day until the
294 fish reached the learning criterion. We combined each set of 12 consecutive trials to one "block" of
295 trials. As we did 6 trials per day, a block lasted at least for two days. To reach the learning criterion,
296 the fish had to first choose the rewarded disc in 10 out of 12 trials (i.e., 80% of correct choices per
297 block) with a maximum of one unsuccessful choice per day. If fish did not reach the learning criterion
298 at the end of a block of 12 trials, the fish entered the next block of 12 trials. This procedure was
299 repeated until the fish either reached the learning criterion or had passed five blocks (60 trials)
300 without reaching the criterion. Once a fish reached the learning criterion in the acquisition phase, we
301 started the reversal phase on the next day. Two fish did not reach the learning criterion and did not
302 proceed to the reversal phase, reducing the sample size to N=56 for the next phase.

303 *(3) Reversal learning phase.* In this phase we reversed the rewarded and unrewarded colour discs, so
304 that the previously unrewarded disc became the rewarded one and vice versa. Otherwise, all
305 procedures and recordings were done as described for the acquisition phase. The reversal phase
306 ended when a fish had reached the learning criterion or after five blocks. All fish tested in the reversal
307 phase reached the learning criterion.

308

309 <u>Hormone sampling</u>

310 After the reversal learning task, we selected 1-2 fish from each group (N=36 fish, Block 1: N=11; Block
311 2: N=13: Block 3: N=12, Supplementary Fig. S1) that had received the food and predator treatments
312 but not the reversal learning task. At the same time all predator presentations stopped, and all
313 groups were transferred to an intermediate food diet (1 stick of JBL Stick M for cichlids® per fish.) The
314 apparatus (see below) enabled the collection of water samples from two fish at the same time. We

315 extended the static holding water method [52] with a continuous water flow that allows the repeated
316 water sampling without further disturbing the fish [41]. The collections for each pair of fish took
317 place over four consecutive days which allowed us to quantify cortisol concentrations (as ng
318 cortisol/L) immediately after a new stressor (i.e. catching and handling of the fish on day 1) as well as
319 cortisol concentrations after fish were habituated to this stressor, i.e. the same catching and handling
320 procedures on day 4 (see below). The involved procedures, including catching, measuring, weighing,
321 and placing the fish in a novel container are perceived as stressful by the experimental animals [41,
322 53]. However, another study showed that *N. pulcher* habituate to this procedure after three days,
323 which allows the collection of cortisol concentrations of fish before and after habituation [41] and
324 thus serves, as a reliable baseline measure.

325 On the first hormone sampling day we caught two fish and measured their body sizes and body
326 masses. We then placed each of the two fish individually in a sampling container filled with 500ml of
327 water and collected four 500ml water samples over 2h. All water samples were collected at the same
328 time in the afternoon (after 14:00h) to control for diurnal variation of cortisol [41]. On two
329 consecutive days, we caught the same two fish and transferred them to the sampling containers filled
330 with 500ml of water for 2h, but did not collect any water samples. This ensured that fish habituated
331 to the catching and handling procedures of the hormone sampling. On the fourth day, we repeated
332 the same procedures, including measuring and weighing the fish and collected another set of four
333 water samples for 2h (see Supplementary Fig. S3). In block 1, due to logistical constraints, we only
334 collected 2 water samples per day, instead of 8, and we decided to always collect the 3rd and 4th water
335 samples on the 1st and 4th day respectively. All other procedures were the same for all three blocks.
336 This resulted in a total of 240 water samples.

337

338 <u>Hormone measurement</u>

339 *Apparatus.* To repeatedly and minimal invasively sample individual cortisol levels, we used a
340 modification of the holding-water method previously used and validated for *N. pulcher* [41]. Exact
341 details of the apparatus can be found in the Supplementary Methods S3. In short, we used a water
342 flow-through system to collect four water samples per fish throughout a 2h sampling period.

343 *Cortisol measures.* Prior analysing cortisol levels, an extraction was performed as following: Water
344 samples were defrosted overnight and filtered in the morning of the next day (paper filter 1/2595
345 grade, diameter 320 mm, Whatman, Sigma-Aldrich, Switzerland) to remove solid particles, and
346 lipophilic compounds were extracted using solid phase extraction (SPE). After the hormone samples

were loaded onto a cartridge (SPE column Isolute C18 (EC), 500 mg/6mL, Biotage, Sweden), the cartridges were stored at - 20 °C until further processing at the University of Vienna.

Conditioned cartridges were defrosted, then placed on a vacuum manifold (Biotage VacMaster 20) and eluted with 5ml ethyl acetate (HPLC -grade 99.9%). Ethyl acetate proved to be a very satisfactory solvent for the elution of cortisol (mainly free CORT) from solid phase extraction cartridges [54]. Collected cortisol samples were dried down with N_2 at 45 °C, resuspend with 750ul assay buffer, vortexed and placed overnight in a fridge. Free cortisol concentrations were quantified using a commercial enzymatic immunoassay for cortisol (ENZO Life Sciences Cat # ADI 900-071). Reconstituted samples were brought to RT on a shaker for 30 min and then further processed following the manufacture's instruction. All samples were analysed in duplicates. The sensitivity of the assay was 24.7 pg/ml, intra-assay CV% was ≤ 5% and inter assay CV% was below 13% using a control sample. Sample concentrations were corrected for dilution. Of the 36 sampled test fish, we were not able to reliably measure cortisol from 2 fish in any of the collected water samples, reducing the overall sample size to 34 fish.

Statistical analysis

For statistical analyses we used R 4.3.3 [55] and the packages lme4 [56], and afex [57]. We used linear mixed effects models (LMM) and generalized linear mixed effects models (GLMM). To obtain p-values, we used either the mixed() function in the package 'afex' with a Satterthwaite's approximation for degrees of freedom, or the drop1() function to do a likelihood ratio test using an F statistics for LMM or an χ^2 statistics for GLMM. Interactions and covariates were stepwise removed if non-significant [58] and reported p-values refer to the final model without non-significant interactions. An overview of all full models is provided in Table S3. To account for familiarity of fish kept in the same group tanks and for potential variations between blocks we always included group tank ID and block ID as random factors.

Based on our predictions and the rationale outlined in the introduction, we aimed to investigate the influence of nutritional state and not feeding regime *per se* on stress reactivity and behavioural flexibility. To compare fish with high and low body condition, we classified them as 'high nutritional state' or 'low nutritional state' depending on whether their body condition was higher or lower than the mean body condition of all fish tested in this study (3.02 ± 0.03 [mean ± SE]). We verified nevertheless that the feeding regimes had the desired effect, and found that fish on the increased diet gained more weight and grew more throughout the experiment than fish on the reduced diet (see Supplementary Table S4, Figs. S6, S7). Consequently, the majority (71%) of fish on the increased

380 diet had above-average body condition and were classified as 'high nutritional state' whereas the
381 majority (74%) of fish on the reduced diet had below average body condition and were classified as
382 'low nutritional state'.
383 To analyse stress response patterns, we first classified each individual as a brief or prolonged
384 responder based on their cortisol pattern in response to the hormone sampling on the first day. Brief
385 responders had shortly elevated cortisol peaks whereas prolonged responders had persistently
386 elevated cortisol concentrations within the 2h sampling period. We used a GLMM with a binomial
387 error distribution to analyse whether the probability to display a brief stress response is influenced by
388 body condition or predator exposures.

389 To estimate the duration of individual stress response patterns, we calculated an index of stress
390 recovery on the first day of sampling. This index was calculated as the difference between the
391 individual peak cortisol concentration and the final value after 2h (at sampling point 4). Thus, a higher
392 recovery value indicates a stress response pattern characterized by a short cortisol peak with a rapid
393 decrease of cortisol within 2h. A lower recovery value indicates a stress response pattern
394 characterised by prolonged periods of high cortisol levels with a slow decrease within 2h. A recovery
395 value of zero indicates a stress response pattern characterised by prolonged periods of high cortisol
396 levels with no decrease within 2h. To assess whether the duration of the stress response is
397 determined by the body condition of the fish, we used a LMM with stress recovery as the dependent
398 variable and the actual body condition as a covariate. We only conducted this analysis after
399 confirming that fish exposed to frequent predators were more likely to show a brief stress response
400 pattern. This result, although not significant, was clearly driven by fish with high body condition (see
401 results). Therefore, to increase the resolution of the post-hoc analyses, we used the stress recovery
402 indices and individual body condition, and focused on the experimental group where the majority of
403 fish showed a brief stress response (i.e. the fish exposed to frequent predators).

404 To analyse behavioural flexibility, we used the number of trials until fish reached the learning
405 criterion in the reversal learning phase as the dependent variable and included body condition ('high',
406 'low´), predator exposure (´no predators´, frequent predators´, ´occasional predators´) as fixed factors
407 in an LMM. To assess whether the treatments influenced the accuracy of fish during the reversal
408 phase we used the proportion of wrong choices over all trials in this phase ('error rate') [59] as the
409 depend variable and included body condition and predator exposure as fixed effects in a GLMM with
410 a binomial error distribution. To assess whether the treatments influenced the fish´s performance
411 during the acquisition phase we used the number of trials and the error rate in a LMM and GLMM
412 respectively with body condition and predator exposure as fixed effects.

413

414 **Results**

415 (1) Do nutritional state and the frequency of stressors determine stress recovery patterns?

416 Contrary to the predictions, the majority of fish exposed to frequent predators had a brief stress
417 response pattern when exposed to a novel stressor, which was not the case for fish exposed to
418 occasional or no predators (Table 1 factor: 'Predator exposure [$\chi2$=8.2, p=0.02], for orthogonal
419 contrasts see Table S5, Fig. 2). In addition, we found that all fish in a high nutritional state that
420 experienced frequent predators showed a brief stress response pattern (see Fig. 2, N=4 out of 4). To
421 test whether body condition determines stress recovery patterns we performed a post-hoc analysis
422 that used the stress recovery index of fish frequently exposed to predators. Although nutritional
423 status did not predict whether fish were classified as brief or prolonged responders (Table 1 factor:
424 'Body condition' [$\chi2$=1.93, p=0.17]), the post-hoc analysis revealed that the magnitude of the stress
425 response, in terms of the individual stress recovery indices, was clearly influenced by body condition
426 (Table 2 factor: 'Body condition' [$F_{1,9}$=8.78, p=0.02]). Fish with a higher body condition had a more
427 rapid recovery pattern than fish with a lower body condition (see Fig. S8). The frequency of predator
428 exposures explained stress response patterns when fish were exposed to a new stressor on day 1 (see
429 Table 1), but not when fish were potentially habituated to the hormone sampling procedures on day
430 4 (Table S6 factor 'Predator exposure' [$\chi2$=1.48, p=0.48]).

431

432 (2) Do nutritional status and the frequency of stressors determine behavioural flexibility?

433 In the reversal learning test, fish took on average 27.2 ± 1.5 and 30.6 ± 1.1 (mean ± SEM) trials to
434 reach the learning criterion in the acquisition and reversal phase, respectively. Two fish did not reach
435 the learning criterion in the acquisition phase and were excluded from the analysis. Behavioural
436 flexibility, in terms of the number of trials until fish reached the learning criterion in the reversal
437 phase, varied depending on the frequency of predator exposures and the nutritional status of the fish
438 (Table 3a factor 'Body condition x Predator exposure' [$\chi2$=5.51, p=0.06]). Contrary to the prediction
439 that frequent stressors and overnutrition would impair behavioural flexibility, orthogonal post-hoc
440 comparisons revealed that fish with high body condition reached the learning criterion faster when
441 exposed to frequent predators compared to fish exposed to occasional predators (Table S7c factor
442 'Occasional predators vs. frequent predators (high body condition)' [estimate: -0.16, z=-3.4, p<0.01,
443 Fig. 3). Predator exposure did not influence behavioural flexibility of fish with a low body condition
444 (see Table S7d,e). Neither predator exposure nor nutritional status influenced the number of trials
445 required to reach the learning criterion in the acquisition phase (Table 3b factors 'Body condition'
446 [χ^2=0.05, p=0.83], 'Predator exposure' [χ^2=0.14, p=0.93]). Similarly, the experimental manipulations

447 did not affect the error rates of the fish during the acquisition phase (Table S8a) and the reversal
448 learning phase (Table S8b).

449

450 **Discussion**

451 Based on the allostatic load model we predicted that overnutrition and frequent exposure to
452 stressors induce prolonged allostatic states including persistently prolonged stress response patterns
453 and impaired behavioural flexibility. Contrary to this prediction, high body condition fish exposed to
454 frequent predators showed brief stress response patterns when exposed to a new stressor. In
455 addition, high body condition fish exposed to frequent predators also showed greater behavioural
456 flexibility. Therefore, the results do not support our initial hypothesis. An alternative interpretation is
457 that our treatments were not sufficient to induce the conditions on which the hypotheses were
458 based: (1) Although fish on the increased diet gained more weight and had more energy resources
459 than fish on the reduced diet, they were not obese, with body masses within the adult range [60],
460 and therefore their condition did not represent overnutrition [6]; (2) The predator presentations
461 clearly induced stress responses in the test fish, however, the frequent predator encounters may not
462 have been enough to induce severe chronic stress. In any case, our results highlight a potential
463 association between brief stress response patterns, characterised by a quick termination of cortisol
464 concentrations, and improved behavioural flexibility measured as reversal learning speed. This
465 association only became apparent when individuals with high body condition, i. e. with more energy
466 reserves available, were exposed to frequent stressors.

467 Glucocorticoid receptors (GR) which are expressed in the brain play an important role in terminating
468 the stress response [61, 62]. A previous study that pharmacologically blocked GRs in *N. pulcher*
469 showed that GR blockade reduced behavioural flexibility in a detour task, highlighting a potential link
470 between stress resilience and the ability to cope with changing conditions. Although cortisol was not
471 measured directly, GR-blocked fish also showed more fear-related behaviours, a possible indication
472 that blocking GRs may have led to an accumulation of cortisol due to the reduced ability to terminate
473 the stress response [63]. Based on the results of the current study, we can conclude that while
474 resilience, defined as an acute stress response, is strongly associated with improved behavioural
475 flexibility, it also comes with associated costs that well-fed individuals are better able to overcome.

476 A prolonged phase of allostasis is the result of a chronic exposure to stressors with high energetic
477 costs and consequences for health and fitness [8], however, the determination of chronic stress load
478 particularly under natural conditions, depends on species-specific factors [64]. We did not find
479 support for the prediction that frequent predator exposures and overnutrition will lead to prolonged

480 allostasis. This prediction was derived from the classic version of the allostatic load model which
481 introduced the concept of allostasis as maintaining homeostastis through change [6]. An extension of
482 the classic version, the reactive scope model, predicts that animals with more energy resources will
483 be better able to cope with repeatedly occurring stressors [8]. This might explain the result that fish
484 with higher body condition had shorter stress response patterns and an improved behavioural
485 flexibility when exposed to frequent predators. Possibly, the higher energetic resources enabled them
486 to express an adequate response to a new stressor, i.e. a brief stress response pattern.

487 Interestingly, fish that had both a high body condition and were exposed frequently to predators also
488 showed more behavioural flexibility. This suggests that the ability to exhibit a stress response pattern
489 with a brief cortisol peak followed by a timely recovery, is associated with higher behavioural
490 flexibility. Stress influences cognition in a complex way that is dependent on the type and duration of
491 the stressor, the individual physiological response type, and the cognitive task being studied [10, 65].
492 Generally, short term stressors are considered to have positive effects on cognitive abilities required
493 to overcome the stressor, whereas long-term or chronic stress is detrimental [65, 66]. Our results
494 provide a valuable contribution to the fields of stress and cognition by highlighting the importance of
495 the nutritional status in determining stress response and behavioural flexibility, i.e. the potential
496 benefits of brief stress response patterns.

497 There are two mutually exclusive explanations as to why fish adapted to the increased frequency of
498 the stressor with beneficial effects on physiology and flexibility. First, fish exposed to frequent
499 predator presentations might have been habituated to the re-occurring stressor leading to shorter
500 stress responses when exposed to a new stressor and with beneficial effects for behavioural
501 flexibility. We took great care to avoid habituation of fish to the predator presentations by varying the
502 timing of the exposures and adding additional stressors such as chasing with a net or creating a
503 moving shadow. Yet, the fish potentially may have developed habituation simply because they were
504 exposed more frequently to the predator presentations. Second, the brief stress responses and
505 improved behavioural flexibility could have been an adaptive response to cope with the frequent
506 predator presentations, which would support the idea of preparative effects of glucocorticoid actions
507 [4, 67, 68]. The 'preparative hypothesis' suggests that prior glucocorticoid actions modulate the
508 response to a subsequent stressor in a mediating or suppressive way [4]. Although not yet tested
509 experimentally, this hypothesis has been used to explain seasonal GC variation. According to the
510 preparative hypothesis both, baseline and stress induced GC levels are higher at challenging times of
511 the year [68, 69]. Yet, stressful periods do not necessarily occur at fixed periods of the year and
512 perceived stress may depend on the social status or demands of the individual [70, 71]. An interesting
513 extension of the preparative hypothesis suggests that GC actions i) prepare to better cope with a

subsequent stressor and ii) increase the threshold when certain stimuli are perceived as a stressor [72]. The observed interaction between body condition and adaptive stress response patterns when exposed to predators is in line with this extension of the hypothesis because the available energy stores affect the ability of organisms to cope with stressors [73].

In conclusion, our results show a potential relationship between stress resilience and behavioural flexibility. Groups of fish that terminated stress responses sooner showed improved behavioural flexibility. This relationship was only seen in groups of fish exposed to frequent predator exposures, which simulated a higher frequency of stressful events. Experiencing more frequent stressful events thus might have prepared fish to better cope with subsequent stressors, particularly in those with greater energy reserves. These results are also consistent with the concept of 'wear and tear', which introduces the idea that frequent exposure to stressors is costly [8] and animals in a better nutritional condition should be better able to cope with repeated stressors due to their higher energy reserves compared to animals in a poor nutritional status. Our results provide direct evidence for the costs of coping with repeated stressors as the manipulation of the nutritional condition interacted with behavioural flexibility. This work is advancing our understanding of the way how animals respond to environmental change.

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Factors	Estimate ± SE	X^2-value	*P*-value
Intercept	-0.47 ± 0.75	-	-
Nutritional state	-1.37 ± 0.89	2.71	0.09
Predator exposure	-*	6	**0.04**

*For estimates please see orthogonal contrasts in Table S3

Table 1: Probability of fish showing acute stress response to a novel stressor. Results are shown from a generalized linear mixed effect model with a binomial error distribution. Predator exposure significantly influenced the probability to show an acute stress response which was irrespective of the nutritional state. Fish were classified as acute or prolonged responders depending on their cortisol concentration profile for two hours on the first water collection day (see methods for more details). In short acute responders decreased cortisol concentrations within two hours whereas prolonged responders did not. Experimental treatments consisted of the independent manipulations of the subject's nutritional state and predator exposure. Nutritional state was included as a two-level factor (high, low) and the estimate is shown as difference to the reference level ('high'). Predator exposure was included as a three-level factor ('no predator', 'occasional predators', frequent predators') and estimates are shown as orthogonal contrasts in Table S3. To obtain p-values a likelihood ration test was used to compare the models with and without the factor of interest. N=34 fish from 20 groups and three blocks. $p<0.05$ is highlighted in bold.

Factors	Estimate ± SE	Num. D.F.	Den. D.F	F-value	*P*-value
Intercept	-28.27 ± 6.86	-	-	-	-
Body condition	9.31 ± 2.35	1	4.78	15.64	**0.01**

Table 2: The intensity of the stress reactivity after experiencing a novel stressor. Results are shown from a linear model with a gaussian error distribution. To obtain normally distributed residuals the dependent variable was log transformed. Fish body condition positively correlated with higher stress reactivity. For each fish cortisol concentrations were measured every 30 minutes (four samples) for two hours on the first water collection day (for more details see methods). The intensity of the stress reactivity was calculated as the difference between the sample showing peak cortisol concentrations and sample four (2h after novel stressor) and a positive value indicates a faster decrease in cortisol concentrations. Fish were exposed to frequent predator encounters and body condition was included as a continuous variable. To obtain p-values a likelihood ration test was used to compare the models with and without the factor of interest. N=34 fish from 20 tanks and 3 blocks. $p<0.05$ are highlighted in bold. N=11 fish from seven groups and three blocks. $p<0.05$ is highlighted in bold

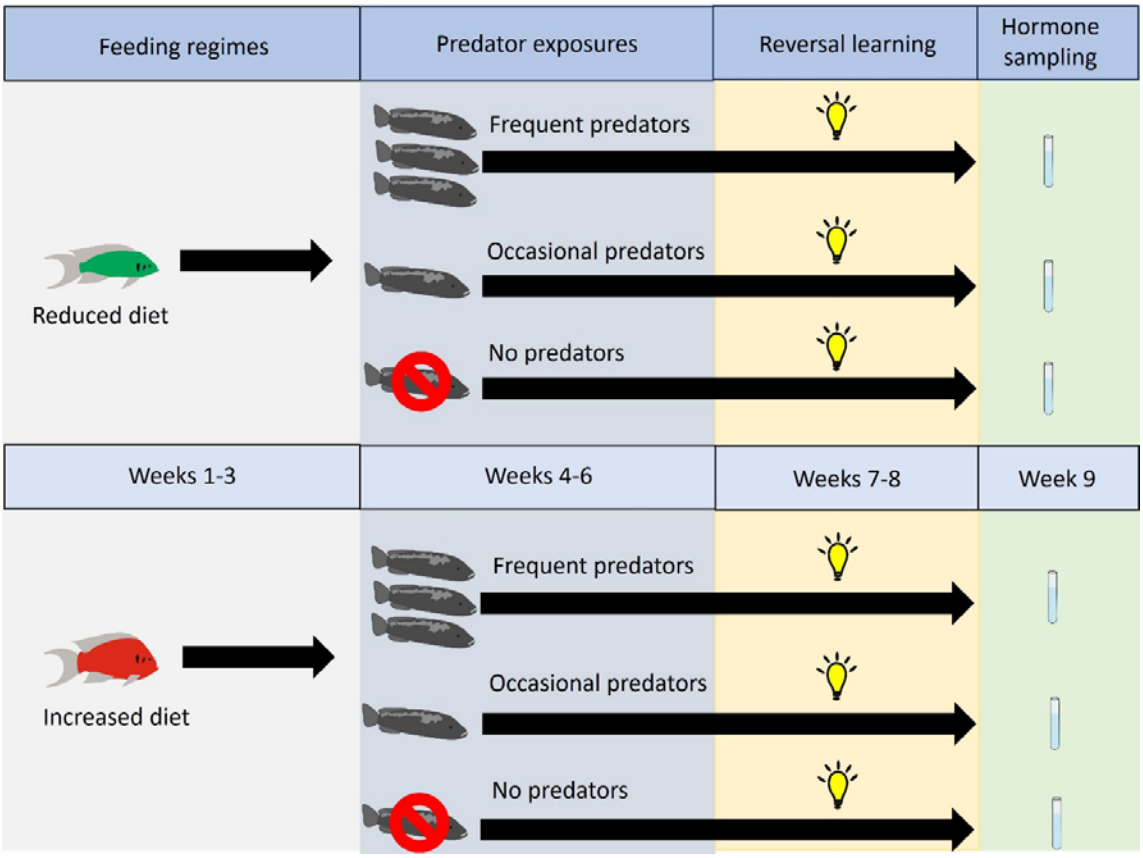
Factors	Estimate ± SE	X^2-value	*P*-value
(a) Number of trials until learning criterion in the reversal phase			
Intercept	3.57 ± 0.07	-	-
Nutritional state	-0.1 ± 0.09	1.45	0.22
Predator exposure	-*	6.23	**0.03**
Body condition x Predator exposure	-*	5.51	*0.05*
(b) Number of trials until learning criterion in the acquisition phase			
Intercept	3.29 ± 0.12	-	-
Nutritional state	0.02 ± 0.12	0.04	0.84
Predator exposure	-	0.23	0.99
Occasional predators	$-6e^{-6}$ ± 0.14	-	-
Frequent predators	0.02 ± 0.14	-	-

*For estimates please see orthogonal contrasts in Table S5

Table 3: The number of trials until fish reached the learning criterion in the (a) reversal and (b) acquisition phase of the learning task. Results are presented from generalized linear mixed effect models with (a) poisson and (b) negative binomial error distributions. Nutritional state and predator exposure interactively influenced the number of trials until fish reached the learning criterion in the reversal but not in the acquisition phase. During the acquisition phase fish had to discriminate between two colours to access a food reward. During the reversal phase the reward consistency was reversed, and fish were again tested until they associated the new colour with the food reward. Experimental treatments consisted of the independent manipulations of the subject's nutritional state and predator exposure. Nutritional state was included as a two-level factor (high, low) and the estimate is shown as difference to the reference level ('high'). Predator exposure was included as a three-level factor ('no predator', 'occasional predators', frequent predators') and estimates in (a) are shown as orthogonal contrasts in Table S5 and in (b) as difference to the reference level 'no predators'. To obtain p-values a likelihood ration test was used to compare the models with and without the factor of interest. N=34 fish from 20 groups and three blocks. $p<0.05$ is highlighted in bold and $p<0.1$ is italicised.

791 **Figure 1**

792

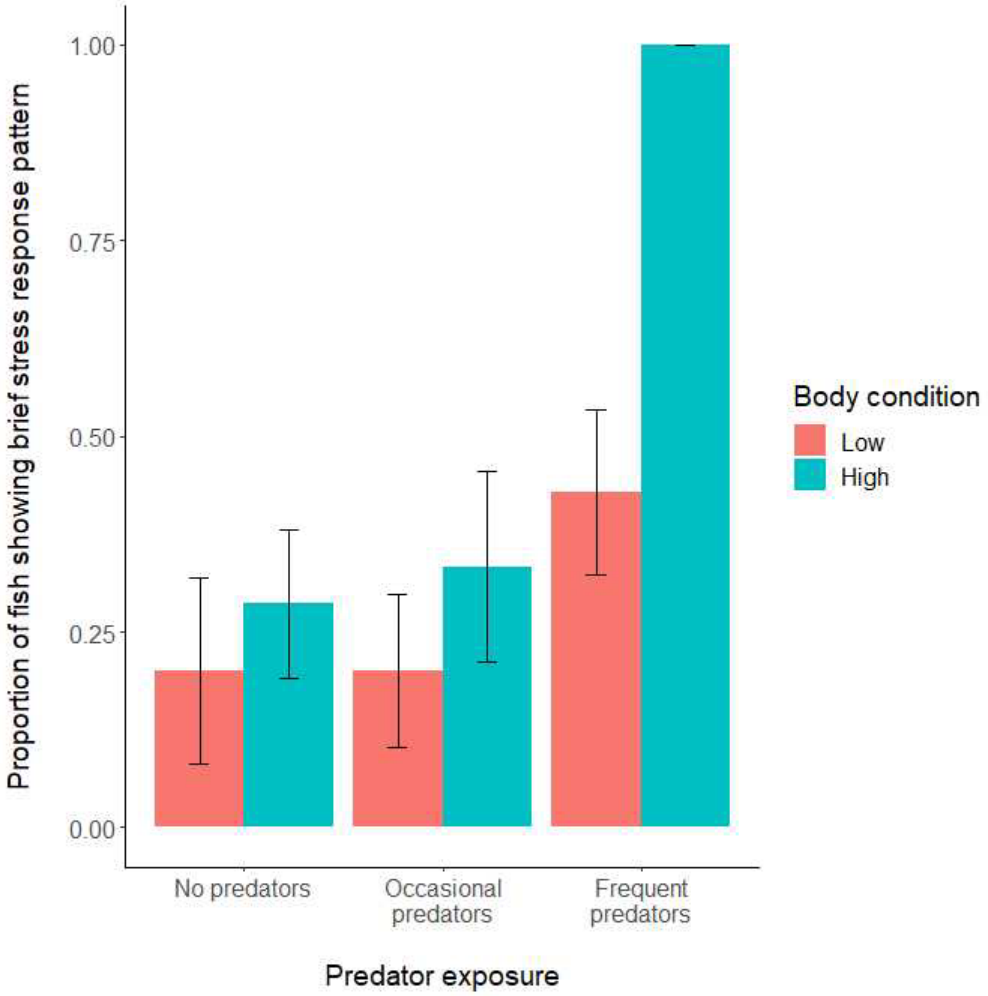


793

794 **Fig. 1**: Timeline of the experiment. The experiment was done in three blocks (not shown) and each
795 block lasted nine weeks which were separated into four phases. In the first phase, fish were kept
796 either on an increased or reduced diet for three weeks. In the second phase, while continuing the
797 feeding regimes, fish were exposed to three different predator exposures: (1) a control exposure
798 without any predators, (2) a occasional or (3) a frequent exposure. In the third phase, we assessed
799 behavioural flexibility using a reversal learning task. In the fourth phase, we collected water samples
800 after exposure to a new stressor to measure stress response patterns in terms of cortisol
801 concentrations.

802

803 **Figure 2**



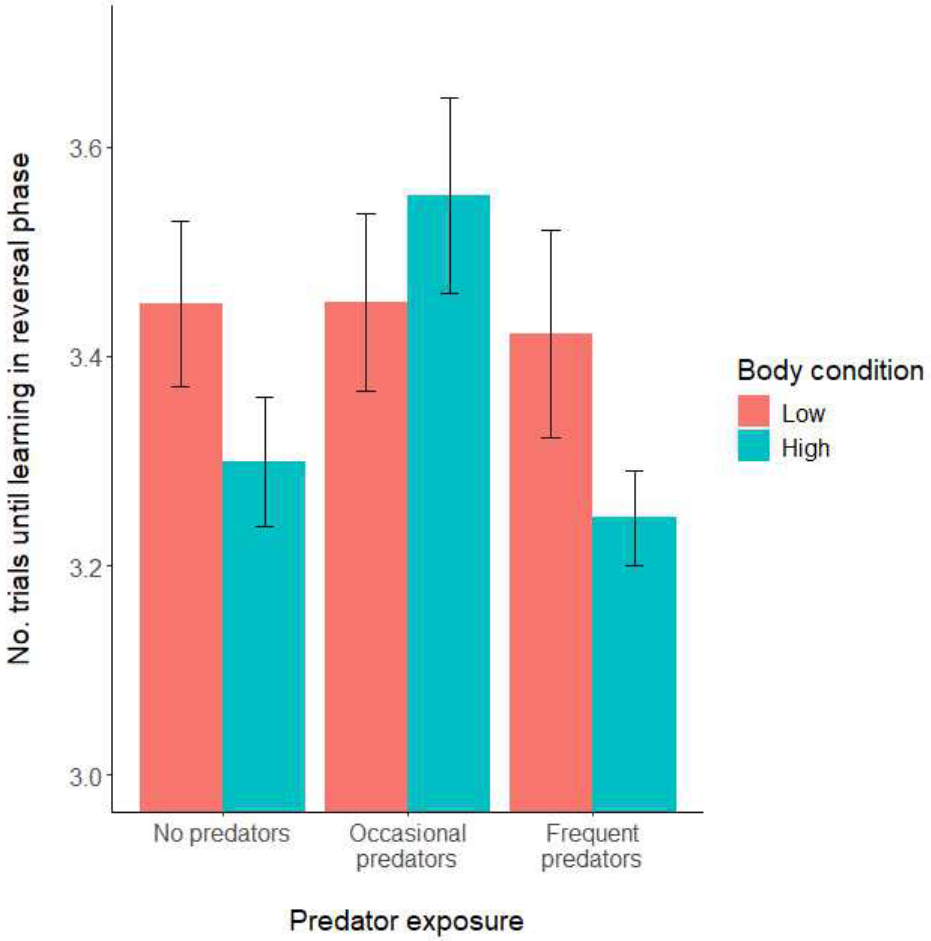
804

805

806 **Fig. 2:** Proportion (mean ± SE) of fish (N=34) showing brief stress response pattern within two hours
807 on the first water sample collection day. Fish were classified as brief or prolonged responders
808 depending on their cortisol concentration profile (see methods for more details). In short brief
809 responders decreased cortisol concentrations within two hours whereas prolonged responders did
810 not. Fish were classified based on their body condition (bc) into: 'low body condition' (red bars, fish
811 with a bc lower than the average) or 'high body condition' (blue bars, fish with a bc higher than the
812 average). Groups were exposed to three predator exposures treatments that included the
813 presentation of a control condition without any predators present ('no predators'), or the
814 presentations of predators with varying frequencies ('occasional predators' or 'frequent predators').
815 Fish exposed to the frequent predator treatment were more likely to show a brief stress response
816 pattern compared to fish exposed to occasional predators. For statistical analysis see Table 1 and
817 Table S3.

818

819 **Figure 3**



820

821 **Fig. 3**: The number of trials (log mean ± SE) until fish reached the learning criterion in the reversal
822 phase of the learning task. Fish were separated into two groups depending on their body condition:
823 low body condition (red bars, lower than the average) or high body condition (blue bars, higher than
824 the average). Groups were exposed to three predator exposure treatments that included the
825 presentation of a control condition without any predators present ('no predators'), or the
826 presentations of predators with varying frequencies ('occasional predators' or 'frequent predators').
827 Fish with a high body condition that were exposed to the frequent predator treatment reached the
828 learning criterion faster than fish with a high body condition that were exposed to the occasional
829 predator treatment. For statistical analysis see Table 3 and Table S5