Active behaviour during early development shapes glucocorticoid reactivity

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Abstract

Glucocorticoids are the final effectors of the stress axis, with numerous targets in the central nervous system and the periphery. They are essential for adaptation, yet currently it is unclear how early life events program the glucocorticoid response to stress. Here we provide evidence that involuntary swimming at early developmental stages can reconfigure the cortisol response to homotypic and heterotypic stress in larval zebrafish (*Danio rerio*), also reducing startle reactivity and increasing spontaneous activity as well as energy efficiency during active behaviour. Collectively, these data identify a role of the genetically malleable zebrafish for linking early life stress with glucocorticoid function in later life.

Introduction

The increased secretion of glucocorticoids like cortisol after the onset of stress (a.k.a.) glucocorticoid reactivity (GC_R) plays a pivotal role in the response to challenge. It is critical for adaptation and central to an organism's resilience[1]. GC_R is a tightly regulated phenomenon, a response of the hypothalamic-pituitary-adrenal (HPA) axis to exogenous or endogenous stressors. Altered functionality of the HPA axis and of GC_R have been associated with detrimental and beneficial consequences for health. They have been linked to stress-evoked disorders including mental disorders as well as increased resilience[2,3,4,5,6,7,8]. Glucocorticoid secretion has been investigated extensively under steady-state and stress conditions[9], and there is ample evidence that HPA axis functionality is susceptible to disturbance by early life stress. Early adversity can, for example, alter glucocorticoid regulation and coping capacities later in life[10,11]. However, it is still unclear how active responses to early life stress can reconfigure HPA axis function, pending a detailed functional evaluation of developmental programming of GC_R. Larval zebrafish are excellent to address this knowledge gap due to their external development, their hypothalamic-pituitary-interrenal (HPI) axis, homologous to the mammalian HPA axis[12], their translucent body, ideal for non-invasive brain imaging and optogenetics[13,14,15], their small size, highly suitable for high-throughput screens with full environmental control, and the availability of tools and methods for identifying genetic and epigenetic modulators, including proteomic technology. Therefore, as a first step, we set out to determine the effect of early life stress on GC_R and coping capacity in larval zebrafish. Taken together our results introduce a high-throughput forced swim test for developing zebrafish and demonstrate that mild early life stress can at least transiently reconfigure GC_R and elicit modulatory adjustments in spontaneous activity and startle reactivity.

Results

High-throughput induction of forced swimming and cortisol increase

Firstly, we exposed groups of larvae to water vortex flows of varying strength, expressed in revolutions per minute (rpm) (for details, see the 'Methods' section). To compare the strength of these flows, we video-recorded and examined the paths (x-y coordinates) of anesthetized larvae (i.e., unable to swim) exposed to vortex flows of increasing rpm (Fig. 1a). The results of these observations confirmed that, as rpm increased, anesthetized larvae followed the vortex currents, thereby moving at higher speeds and larger distances from the source of the vortex. These measurements were used to determine vortex flows of low, medium and high strength. We then assessed the relationship between the strength of the flows and the behaviour of freely swimming larvae. Larval zebrafish have been shown to display positive rheotaxis[16,17], i.e., spontaneous swimming against an oncoming current, which allows them to hold their position instead of being swept downstream by the current. When exposed to vortex flows, freely swimming larvae held their position away from the vortex's source, thereby avoiding the strongest currents (Fig. 1b, Kruskal-Wallis test, H=27.6, $p < 0.0001$, followed by Dunn's multiple comparison tests). They also faced the oncoming current (Fig. 1c, top, Chi-square test, $X^2(1, N=270)=55.4$, $p < 0.0001$) and adjusted their swim bouts and turns (Fig. 1c, bottom, one-way ANOVA, F(2,29)=6.7, $p = 0.004$, followed by *post hoc* comparisons) to compensate for vortex strength. As indicated by their GC_R, these behaviours were taxing for the larvae. Their whole-body cortisol increased together with the strength of the vortex (Fig. 1d, one-way ANOVA, F(2,17)=35.4, $p < 0.0001$, followed by *post hoc* comparisons).

Cortisol change in response to water vortex flows as a function of development

Secondly, we assessed GC_R to vortex flows as a function of development, expressed in days post fertilization (dpf). For these tests we selected vortex flows of medium strength (i.e., 330 rpm), avoiding the high strength causing maximum levels of vortex-dependent cortisol increase and occasional disruptions of positive rheotaxis (not shown). The HPI axis of

zebrafish matures early. Basal whole-body cortisol and expression levels of genes involved in corticosteroid synthesis and signaling increase drastically around the time of hatching[18,19]. We observed that whole-body cortisol increased gradually between 2 and 8 dpf (Fig. 2a, top, Kruskal-Wallis test, H=64.7, $p < 0.0001$, followed by Dunn's multiple comparison tests), and that the magnitude of the vortex-dependent elevation of cortisol peaked at 6 dpf (Fig. 2a, bottom, Kruskal-Wallis test, H=36.5, $p < 0.0001$, followed by Dunn's multiple comparison tests), with circulating levels of cortisol measured ten minutes after a three minute exposure to vortex flows (for details, see the 'Methods' section). Mechanistically, this points to fundamental alterations in the HPI axis occurring at 4-6 dpf.

Prolonged forced swimming and HPI activation at 5 dpf

Thirdly, building on the above findings, we exposed 5 dpf larvae to vortex flows of medium strength for 9 hours. Exposed larvae showed increased levels of whole-body cortisol which peaked shortly after the onset of the vortex and remained high four hours later compared with controls, i.e., unexposed larvae that were equally handled, but the vortex flows were not present. Both exposed and control larvae showed similar levels of whole-body cortisol six hours after the onset of the vortex (Fig. 2b, Kruskal-Wallis tests, exposed larvae: H=36.9, $p < 0.0001$, control larvae: H=36.3, $p < 0.0001$, followed by two-group comparisons using Mann-Whitney tests). Also, exposed larvae remained engaged in positive rheotaxis from the beginning to the end of the vortex, as indicated by their δ body angle (average change in orientation after a swim bout) measured 5 minutes (Fig. 2c, top, two-tailed *t*-test, t(32)=3.8, $P = 0.0007$) and 8.5 hours (Fig. 2c, bottom, two-tailed *t*-test, t(32)=4.1, $P = 0.0003$) after the onset of vortex flows (see also the 'Methods' section).

Increased baseline swimming and reduced startle reactivity in pre-exposed larvae

Next, using video-recordings and off-line measurements (for details, see the 'Methods' section), we assessed the behaviour of 6 dpf larvae that had or had not been exposed to vortex flows at 5 dpf, i.e., pre-exposed and control larvae, respectively. Compared with controls, pre-exposed larvae showed higher levels of baseline swimming (Fig. 2d, top, two-tailed *t*-test, t(41)=2.2, $P = 0.03$) and reduced startle reactivity upon re-exposure to vortex flows of medium strength, as specified by the distance they swam directly after the onset of the water current (Fig. 2d, bottom, two-tailed *t*-test, t(24)=2.7, $P = 0.01$). Importantly, pre-exposed larvae engaged in positive rheotaxis as efficiently as controls, as indicated by the proportion of larvae facing the oncoming current (Fig. 2e, top, Chi-square test, $X^2(1, N=180)=1.4$, $p = 0.24$) and δ body angle (Fig. 2e, bottom, two-tailed *t*-test, t(18)=0.08, $P = 0.94$).

Reduced glucocorticoid reactivity to vortex flows in pre-exposed larvae

At 6 dpf, pre-exposed larvae, which had prior experience with the vortex flows at 5 dpf, displayed the above behavioural adjustments as well as reduced GC_R. Relative to controls, pre-exposed larvae showed similar levels of basal cortisol and reduced levels of vortex-dependent cortisol increase upon re-exposure to vortex flows of medium strength (Fig. 2f, Kruskal-Wallis tests, pre-exposed larvae: H=15.2, $p = 0.0005$, control larvae: H=13.7, $p = 0.001$, followed by two-group comparisons using two-tailed *t*- or Mann-Whitney tests, basal cortisol: t(10)=0.48, $P = 0.65$, 10 minutes after exposure: U=2, $P = 0.01$, 20 minutes after exposure: t(10)=4.0, $P = 0.003$). We found the same pattern of results at 10 dpf (Fig. 2g, two-way ANOVA, group: F(1,30)=32.8, $p < 0.0001$, time: F(2,30)=40.5, $p < 0.0001$, group x time: F(2,30)=5.6, $p = 0.009$, followed by *post hoc* comparisons).

Short-term reduced glucocorticoid reactivity to heterotypic stress

To complement these assessments we examined the relationship between GC_R in pre-exposed larvae and heterotypic stress. For this we exposed 6 dpf pre-exposed and control larvae to hyperosmotic medium (NaCl), a known stress protocol (for details, see the 'Methods' section). The results showed that, relative to controls, pre-exposed larvae showed reduced GC_R to moderate and high levels of salt stress (Fig. 2h, left, two-way ANOVA, group: $F(1,20)=51.0$, $p < 0.0001$, NaCl concentration: $F(1,20)=299.0$, $p < 0.0001$, group x NaCl concentration: $F(1,20)=0.3$, $p = 0.59$, followed by *post hoc* comparisons). By contrast, at 10 dpf, both groups showed similar cortisol responses to salt stress (Fig. 2h, right, two-tailed *t*-test, $t(10)=0.9$, $P = 0.42$).

Discussion

Hormones react to the environment and cause changes in physiology as a function of maturation. Thus the question arises as to how dynamical patterns of hormone secretion are achieved and what effects they exert on well-being. How does the environment activate and guide the development of resilience mechanisms? Current paradigms stipulate that glucocorticoids are fundamental to the mitigation of allostatic load[9,20]. However, the impact of early life stress on developmental programming of GC_R has not been explored in full, in part due to a lack of suitable models. We now show in zebrafish that the increase in cortisol elicited by a brief period of involuntary swimming peaks at 6 days post fertilization (dpf), pointing to fundamental changes in GC_R at early larval stages. Importantly, we found that, if prolonged for hours, forced swimming at 5 dpf caused a transient form of hypercortisolaemia and later led to reduced GC_R. If subsequently exposed to a brief period of involuntary swimming (i.e., homotypic stress), pre-exposed larvae showed a decreased cortisol response that persisted for at least five more days. Moreover, twenty-four hours after prolonged forced swimming at 5 dpf, the reduced GC_R appeared invariant to stressor identity, as indicated by a decreased cortisol response to heterotypic stress, i.e., osmotic shock. These data suggested

149 that the sustained reduction in GC_R did not reflect a process of habituation to sensory input[21].
150 It seems likely that, in pre-exposed larvae, reduced GC_R was underpinned by changes in state
151 variables of the HPI axis.

152 Early stress as well as chronic stress in later life can decrease hypothalamic activity
153 and expression of corticotropin-releasing-hormone (CRH) and arginine-vasopressin (AVP).
154 These changes are mediated at least in part by glucocorticoids[22,23,24,25]. Previous studies in fish
155 showed that prolonged stimulation of the HPI axis can attenuate the stress response[26]. This
156 effect can result from transcriptional regulation of CRH and adrenocorticotropic hormone
157 (ACTH)[27,28,29]. Additionally, pituitary corticotrophs and cortisol-producing cells in the
158 interrenal gland may be desensitized to CRH or ACTH, respectively[30,31]. Stressor exposure on
159 early stages of rainbow trout can lead to HPI axis hypoactivity later in life[32]. In zebrafish,
160 incubation in cortisol during the first 48 hours post fertilization caused altered locomotor
161 reactions to photic stimuli[33]. Also, incubating zebrafish embryos in cortisol during the first
162 five days post fertilization caused increased whole-body cortisol, glucocorticoid signalling
163 and expression of immune-related genes; these changes can be long-lasting and result in
164 dysfunctional capacities for regeneration along with increased expression of inflammatory
165 genes[34]. A similar treatment using dexamethasone also induced long-lasting behavioural and
166 metabolic changes still detectable in adulthood[35]. Further experiments are necessary to
167 determine whether reduced GC_R in pre-exposed larvae occurs via receptor downregulation,
168 decreased synthesis and/or depletion of hormones, and/or increased sensitivity to
169 glucocorticoid feedbacks[36,37,38].

170 An organism is said to be engaged in active behaviour when it is the source of the
171 output energy required for a given action[39]. Glucocorticoids are known to mobilize energy[1],
172 necessary to face the demands of forced swimming. In response to the vortex, larvae engaged
173 in rheotaxis had to adjust their swim bouts and turns continuously to compensate for the
174 oncoming current. These actions were energy demanding for the larvae, as revealed by their

175 GC_R. The notion that upholding positive rheotaxis for hours involved mobilizing energy was
176 supported by the long-lasting hypercortisolic state observed during prolonged forced
177 swimming. Twenty-four hours after prolonged exposure to the vortex, we observed
178 differences between pre-exposed and control larvae. Firstly, pre-exposed larvae displayed
179 increased levels of baseline swimming. A previous study in zebrafish showed that involuntary
180 swimming at larval stages can subsequently increase spontaneous activity[40]. Secondly, upon a
181 brief re-exposure to the same vortex, pre-exposed larvae showed reduced startle reactivity to
182 the onset of water motions. Thirdly, they engaged in positive rheotaxis as efficiently as
183 controls. On the assumption that the cortisol response to the vortex reflects an energy
184 requirement for positive rheotaxis, these observations indicated that pre-exposed larvae
185 responded more efficiently to the energy demands of forced swimming.

186 In conclusion, we have shown in larval zebrafish that early life stress caused by
187 prolonged forced swimming at least transiently reconfigures the increased secretion of
188 cortisol after the onset of homotypic or heterotypic stress, as well as spontaneous activity and
189 efficient energy use during active behaviour. It remains open how these changes relate to
190 survival in a species facing greater mortality during early life; there is a lack of evidence
191 linking early activity patterns of the HPI axis to survival and reproductive outcome.
192 Collectively, our data provided direct evidence to support the contention that long-term
193 changes in HPI axis function after early adversity may later lead to increased resilience in
194 developing zebrafish. Increased resilience to stress may have advantages for larval zebrafish.
195 Such ability may help larvae to better cope with antagonistic environments. An important
196 question emerging relates to the study of stress reactivity during adulthood as a function of
197 early life events in zebrafish. A previous study in mice reported that individuals that had
198 endured early life stress coped better with forced swimming compared with those that had
199 experienced a favourable early care regime[41]. In adult rats, the adverse experience of maternal
200 separation during early life strengthened freezing during fear conditioning after chronic stress

compared with non-maternally separated rats[42]. These studies support the view that early life stress can subsequently lead to increased resilience. Long-lasting changes in HPA axis function due to early experiences have been attributed to changes in the epigenome[43], although the link between early life stress and the activation of resilience mechanisms has been difficult to pin down in models with intrauterine development. In zebrafish, all three elements of the HPI axis can be visualized and genetically manipulated at early developmental stages and measured with modern molecular tools[13]. Moreover, the larval brain is readily accessible and provides excellent access for assessing how systematic variations in physiological and behavioural schemes relate to differences in the activity of neuronal and humoral networks. Further studies are required to determine the applicability of our high-throughput procedure. In anticipation to these studies, we speculate that zebrafish larvae will prove fruitful to link early HPI axis activity to proteomic regulation, epigenetic programming and measures of stress resilience.

Methods

Zebrafish husbandry and handling

Zebrafish breeding and maintenance were performed under standard conditions[44]. Groups of thirty wild-type embryos (cross of AB and TL strains, AB/TL) were collected in the morning and raised on a 12:12 light/dark cycle at 28 °C in 35 mm Petri dishes with 5 ml of E2 medium. At 3 days post fertilization (dpf), the E2 medium was renewed and chorions and debris were removed from the dishes. Experiments were carried out with 5-6 dpf larvae, with the exception of the cortisol measurements in Fig. 2a, g and h(left). Larvae older than 6 dpf were transferred to plastic cages with 400 ml of egg water in groups of thirty and fed with paramecia daily. Tests were performed between 09:00 hours and 18:00 hours, with different experimental groups intermixed throughout the day. Zebrafish experimental procedures were

226 performed according to the guidelines of the German animal welfare law and approved by the
227 local government (Regierungspräsidium Karlsruhe; G-29/12).
228 *Water vortex flows*
229 Water current can trigger rheotaxis in larval zebrafish and, if sufficiently strong, it can also act
230 as a stressor, causing a sharp increase in whole-body cortisol via the activation of the HPI
231 axis. We used water vortex flows in a high-throughput fashion to induce both rheotaxis and
232 cortisol increase. For this we exposed groups of thirty (4-8 dpf) larvae in 35 mm Petri dishes
233 with 5 ml of E2 medium to the vortex flows caused by the spinning movements of small
234 magnetic stir bars (6 x 3mm, Fischerbrand, #11888882, Fisher scientific, Leicestershire, UK.)
235 inside the dishes. The Petri dishes, each with a single stir bar, were positioned on magnetic
236 stirrer plates (Variomag, Poly 15; Thermo Fisher Scientific, Leicestershire, UK) and kept at
237 28°C inside an incubator (RuMed 3101, Rubarth Apparate GmbH, Laatzen, Germany).
238 Larvae were presented with either short (3 minutes) or long (9 hours of continuous
239 stimulation) exposure periods to the vortex flows caused by the highly-controlled magnetic
240 field inversions of the stirrer plate, of 130, 330 or 530 revolutions per minute (rpm). For the
241 short exposure, we avoided exposure periods longer than 3 minutes to elude maximum levels
242 of stressor-mediated cortisol increase (not shown). The long exposure at 5 dpf consisted of 9
243 hours to achieve the longest possible exposure period adjustable to the light/dark cycle. Once
244 exposed, larvae were immobilized in ice water and used for cortisol measurement (see below).
245 Control larvae were collected after equal handling, omitting exposure to vortex flows (i.e., stir
246 bars inside the Petri dishes were absent). To rule out unspecific effects of the magnetic field
247 inversions produced by a stirrer plate, we compared the level of basal whole-body cortisol
248 across groups of 6 dpf larvae that either remained unexposed or had been exposed to magnetic
249 field inversions alone (i.e., without stir bars inside the Petri dishes and thus in the absence of
250 vortex flows), of 130, 330 and 530 rpm. The results of these tests showed that magnetic field

251 inversions per se did not alter the level of whole-body cortisol (one-way ANOVA,
252 $F(3,23) = 0.05$, $p = 0.98$).
253 *Re-exposure to vortex flows*
254 For these tests we selected vortex flows of medium strength to avoid possible ceiling effects
255 caused by maximum levels of vortex-dependent cortisol increase. Using the above protocol,
256 larvae that had or had not been exposed to vortex flows for 9 hours at 5 dpf were re-exposed
257 to vortex flows (330 rpm) for 3 minutes at either 6 or 10 dpf. They were subsequently used
258 for cortisol detection or behaviour evaluation.
259 *Re-exposure to vortex flows at 10 dpf*
260 A plastic cage (5 L) containing thirty pre-exposed or control 10 dpf larvae and three magnetic
261 stir bars (25 x 6 mm, Fisherbrand, #10226853, Fisher scientific, Leicestershire, UK)
262 distributed equidistantly along the bottom of the cage were placed on top of the magnetic
263 stirrer plate (Variomag, Poly 15, Thermo Scientific, Leicestershire, UK). Larvae were then
264 exposed to vortex flows (330 rpm) for 3 minutes. Larvae were then immobilized with ice
265 water and used for cortisol extraction 10 minutes after the onset of the vortex flows.
266 *Hyperosmotic medium*
267 Groups of thirty larvae (either 6 or 10 dpf) in 35 mm Petri dishes were incubated for 10 min in
268 steady state E2 medium (controls) or E2 + 50 or 250 mM NaCl (Merck, #106404, Darmstadt,
269 Germany) at 28°C under white light illumination. They were washed three times with E2
270 medium and kept for immediate cortisol detection. The wash and transfer period took 3 min
271 (± 10 s) and was performed at room temperature.
272 *Whole-body cortisol*
273 The procedures and the home-made ELISA used for cortisol measurements were as
274 previously described[13,45]. Groups of thirty larvae were immobilized in ice water after being
275 exposed to water vortex flows or NaCl. Unexposed larvae (control samples) were collected
276 after equal handling, omitting stressor exposure. Samples were then frozen in an ethanol/dry-

277 ice bath and stored at −20 °C for subsequent extraction. Each replicate consisted of a well
278 with 30 larvae. Cortisol extraction and detection were carried between 10:30 and 11:30 hours.
279 *Independent sampling*
280 The scheme of independent sampling has been described elsewhere[46]. Briefly, cortisol and
281 behavioural measurements were made on different groups of equally treated larvae and
282 therefore constitute fully independent samples. For the behavioural measurements, each
283 replicate involved a single larva. Yet, these individual measurements were made on larvae
284 that had also been kept in wells containing a total of thirty larvae per well. Thus, the number
285 of single larvae matched the number of independent wells. In this manner, the density of
286 larvae per well during vortex flow exposure remained a constant factor for both the cortisol
287 and behavioural measurements. For each cortisol measurement, all thirty larvae in a well were
288 used, whereas each behavioural measurement involved only one larva, the remaining twenty-
289 nine larvae in the well were used elsewhere. Each replication was fully independent from the
290 others thus avoiding pseudo-replication.
291 *Anesthetized larvae*
292 To assess the speed and trajectories of anesthetized larvae exposed to vortex flows of
293 increasing strength, 6 dpf larvae in 35 mm Petri dishes were first incubated in 5 mL of steady
294 state E2 medium + 100 µL of Tricaine (Sigma-Aldrich #E10521, Schnelldorf, Germany); they
295 were considered to be anesthetized when they failed to respond to tactile stimulation. They
296 were then transferred to a new Petri dish with fresh E2 medium (5 mL) for testing.
297 *Behaviour evaluation*
298 Video recordings were conducted under conditions identical to those of the cortisol
299 measurements. Groups of thirty larvae (either 5 or 6 dpf, depending on the experiment) were
300 imaged at 12.5 frames s^{-1} with a camera (HDR-CX240 HD Flash, Sony, Berlin, Germany)
301 positioned above a 35 mm Petri dish with 5 ml of E2 medium and a magnetic stir bar placed
302 on a magnetic stirrer plate inside the incubator, as described above. Videos samples were later

303 used for offline data recovery using ImageJ 1.48v software (National Institutes of Health,
304 Bethesda, USA) and MTrackJ (Biomedical Imaging Group Rotterdam, Rotterdam, The
305 Netherlands). Larvae were individually tracked and their x-y coordinates at every time point
306 were subsequently used to calculate motion values, body orientation and position relative to
307 the rotation axis of the magnetic stir bar, which corresponded in all cases to the center of the
308 Petri dish. Motion values were expressed as either speed (mm per second) or distance swum
309 every 5 or 30 seconds. To quantify the proportion of larvae engaged in rheotaxis, we
310 measured the proportion of larvae directly facing the oncoming current 120 s after the onset
311 of vortex flows. For this we measured - three times every 10 s - the angle formed between a
312 larva's body axis and a line connecting the center of its head and the rotation axis of the
313 magnetic stir bar. A larva was considered to be engaged in rheotaxis when the coefficient of
314 variation arising from the three angles measured over 30 s remained lower than 10 % and, at
315 the same time, it exhibited minimum body displacements, i.e., shorter than 0.5 mm * (10
316 ms)$^{-1}$. To assess the average change in orientation after a swim bout (δ body angle, in
317 degrees), we measured, as before, the angle formed between a larva's body axis and a line
318 connecting the center of its head and the rotation axis of the magnetic stir bar, every 933 ms
319 over a 5 s period 120 s after the onset of vortex flows. The resulting 'δ body angle' values (in
320 degrees) were then calculated as the average difference between the consecutive angles for
321 each larva.

322 *General experimental design and statistical analyses*

323 In all experiments, replicates were randomly assigned with a treatment using a computer-
324 generated random number sequence. Also, we used blinding. For cortisol measurements, a
325 first experimenter executed the treatments and collected and labelled the samples. A second
326 experimenter extracted cortisol from the labelled samples and assigned them with new labels.
327 The first experimenter then quantified cortisol using the newly encoded samples. The results
328 were compared by the first, second and a third experimenter at the end of the blind procedure.

329 For behavioural measurements, a first experimenter performed the video-recordings and
330 labelled the resultant test videos. A second experimenter then scored the behaviour of single
331 larvae in each labelled video. Thus all videos were assessed blind. At the end of the blind
332 procedure, the identities of the larvae were linked to the treatments and the results examined
333 collectively in the presence of a third experimenter. In our study, all data are shown as single
334 measurement points or mean and standard error of the mean. No data points or samples were
335 excluded from analysis. Our sample sizes are generally employed in the field and based on
336 previous publications[13,45], statistical methods to predetermine sample size were not necessary.
337 The sample sizes in Figs. 1b, 1c (top and bottom), 1d and 2a (top and bottom) are unbalanced
338 due to the scale of the overall study (only partially shown) and the ensuing number of
339 simultaneous groups. In our study, several experiments were being carried out in parallel. As
340 a result, the amount of effort that could be devoted to each one of them varied over time. We
341 therefore set up a minimum sample size for each dataset from the very beginning, based on
342 our previous publications and experience. Whenever likely, we added as many data points as
343 possible, depending on the parallel experiments. Data points could have been randomly
344 selected so as to keep sample size constant across all groups. Yet the unbalanced samples did
345 not preclude the validity of the applied tests, they constituted independent samples and
346 pairwise comparisons remained valid in all cases. We decided against the exclusion of data
347 points to favour a better estimate of the coefficient of variation of the various samples. The
348 values in Figs. 1c (top) and 1c (bottom) arise from the same type of treatment, but do not
349 necessarily involve the same set of animals. Fig. 1c top depicts a fraction (percentage) of
350 larvae from a group that involved several trials; here sample size refers to the total number of
351 larvae in several dishes across trials. By contrast, sample size in Fig 1c bottom refers to
352 individuals. Normality was tested using the Shapiro–Wilk test. Normally distributed data
353 were then tested for homogeneity of variance using Bartlett's tests. For two-group
354 comparisons, we used Mann-Whitney tests (for pairwise comparisons with at least one non-

normally distributed dataset) and Student's *t*-tests (two-tailed) (normally distributed data). For multiple group comparisons we used Chi-square tests, as well as Kruskal-Wallis tests and ANOVAs, followed by Dunn's multiple comparison tests and Bonferroni's *post hoc* tests, respectively. Analyses were made with MS-Excel (Microsoft Corp; Redmond, WA, USA), Prism 5 (Graphpad Software Inc, San Diego, CA, USA), ImageJ (Freeware) and VirtualDub (Freeware). The figures were assembled in Illustrator (Adobe).

Data accessibility

The data that support the findings of this study are available from the authors on request.

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Author Contributions

Conceptualization, R.J.D.M. and S.R.; Methodology, R.J.D.M. and S.R.; Investigation, L.A.C-R., S.R. and R.J.D.M.; Writing – Original Draft, L.A.C-R and R.J.D.M.; Writing – Review & Editing, R.J.D.M.

Competing Interests

The authors declare no competing (financial and non-financial) interests.

References

1. Sapolsky, R. M., Romero, L. M. & Munck, A. U. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* **21**(1), 55-89 (2000).
2. Daskalakis, N. P., Bagot, R. C., Parker, K. J., Vinkers, C. H. & de Kloet, E. R. The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinol.* **38**(9), 1858-1873, https://doi:10.1016/j.psyneuen.2013.06.008 (2013).
3. Khulan, B. & Drake, A. J. Glucocorticoids as mediators of developmental programming effects. *Best Pract. Res. Clin. Endocrinol. Metab.* **26**(5), 689-700, https://doi:10.1016/j.beem.2012.03.007 (2012).
4. McEwen, B. S. Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *Eur. J. Pharmacol.* **583**, 174–185 (2008).
5. Nederhof, E. & Schmidt, M. V. Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. *Physiol. Behav.* **106**(5), 691-700, https://doi:10.1016/j.physbeh.2011.12.008 (2012).
6. Reynolds, R. M. Glucocorticoid excess and the developmental origins of disease: two decades of testing the hypothesis--2012 Curt Richter Award Winner. *Psychoneuroendocrinol*. **38**(1), 1-11, https://doi:10.1016/j.psyneuen.2012.08.012 (2013).
7. Schmidt, M. V. Molecular mechanisms of early life stress--lessons from mouse models. *Neurosci. Biobehav. Rev.* **34**(6), 845-852, https://doi:10.1016/j.neubiorev.2009.05.002 (2010).
8. Seckl, J. R. & Meaney, M. J. Glucocorticoid programming. *Ann. N. Y. Acad. Sci.* **1032**, 63-84, https://doi:10.1196/annals.1314.006 (2004).

9. Reul, J. M. H. M., Collins, A., Saliba, R. S., Mifsud, K. R., Carter, S. D., Gutierrez-Mecinas, M., Qian, X., Linthorst, A. C. E. Glucocorticoids, epigenetic control and stress resilience. *Neurobiol. Stress* **1**, 44-59, https://doi:10.1016/j.ynstr.2014.10.001 (2014).
10. Russo, S. J., Murrough, J. W., Han, M., Charney, D. S. & Nestler, E. J. Neurobiology of Resilience. *Nat. Neurosci.* **15**(11), 1475-1484, https://doi:10.1038/nn.3234 (2012).
11. Strüber, N., Strüber, D. & Roth, G. Impact of early adversity on glucocorticoid regulation and later mental disorders. *Neurosci. Biobehav. Rev.* **38**, 17–37, https://dx.doi.org/10.1016/j.neubiorev.2013.10.015 (2014)
12. Wendelaar Bonga, S. E. The stress response in fish. *Physiol. Rev.* **77**(3), 591-625 (1997).
13. De Marco, R. J., Thiemann, T., Groneberg, A. H., Herget, U. & Ryu, S. Optogenetically enhanced pituitary corticotroph cell activity post-stress onset causes rapid organizing effects on behaviour. *Nat. Commun.* **7**, 12620, https://doi:10.1038/ncomms12620 (2016).
14. Gahtan, E. & Baier, H. Of lasers, mutants, and see-through brains: functional neuroanatomy in zebrafish. *J. Neurobiol.* **59**, 147–161 (2004).
15. Portugues, R., Severi, K. E., Wyart, C. & Ahrens, M. B. Optogenetics in a transparent animal: circuit function in the larval zebrafish. *Curr. Opin. Neurobiol.* **23**, 119–126 (2013).
16. Olszewski, J., Haehnel, M., Taguchi, M. & Liao, J. C. Zebrafish larvae exhibit rheotaxis and can escape a continuous suction source using their lateral line. *PLoS One* **7** https://doi: 10.1371/journal.pone.0036661 (2012).
17. Suli, A., Watson, G. M., Rubel, E. W. & Raible, D. W. Rheotaxis in larval zebrafish is mediated by lateral line mechanosensory hair cells. *PLoS One* **7**:e29727, https://doi: 10.1371/journal.pone.0029727 (2012).
18. Alsop, D. & Vijayan, M. M. Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **294**(3), 711-719, https://doi:10.1152/ajpregu.00671.2007 (2008).

19. Alderman, S. L. & Bernier, N. J. Ontogeny of the corticotropin-releasing factor system in zebrafish. *Gen. Comp. Endocrinol.* **164**, 61–69 (2009).

20. McEwen, B.S. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. *Ann. N. Y. Acad. Sci.* **933**, https://doi.org/10.1111/j.1749-6632.2001.tb05830.x (2001).

21. Grissom, N. & Bhatnagar, S. Habituation to repeated stress: get used to it. *Neurobiol. Learn. Mem.* **92**(2), 215-224, https://doi:10.1016/j.nlm.2008.07.001 (2009).

22. Erkut, Z. A., Pool, C. & Swaab, D. F. Glucocorticoids suppress corticotropin-releasing hormone and vasopressin expression in human hypothalamic neurons. *J. Clin. Endocrinol. Metab.* **83**(6), 2066-2073, https://doi:10.1210/jcem.83.6.4881 (1998).

23. Herman, J. P., Flak, J. & Jankord, R. Chronic stress plasticity in the hypothalamic paraventricular nucleus. *Prog. Brain Res.* **170**, 353-364, https://doi:10.1016/s0079-6123(08)00429-9 (2008).

24. Tasker, J. G. & Herman, J. P. Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic-pituitary-adrenal axis. *Stress* **14**(4), 398-406, https://doi:10.3109/10253890.2011.586446 (2011).

25. Wismer Fries, A. B., Ziegler, T. E., Kurian, J. R., Jacoris, S. & Pollak, S. D. Early experience in humans is associated with changes in neuropeptides critical for regulating social behavior. *Proc. Natl. Acad. Sci. U.S.A.* **102**(47), 17237-17240, https://doi:10.1073/pnas.0504767102 (2005).

26. Barton, B. A. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.,* **42,** 517-25 (2002).

27. Birnberg, N. C., Lissitzky, J. C., Hinman, M. & Herbert, E. Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion. *Proc. Natl. Acad. Sci. USA.* **80**(22), 6982-6986 (1983).

28. Eberwine, J. H. & Roberts, J. L. Glucocorticoid regulation of pro-opiomelanocortin gene transcription in the rat pituitary. *J. Biol. Chem.* **259**(4), 2166-2170 (1984).

29. Imaki, T., Nahan, J. L., Rivier, C., Sawchenko, P. E. & Vale, W. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *J Neurosci.* **11**(3), 585-599 (1991).

30. Hontela, A., Rasmussen, J. B., Audet, C. & Chevalier, G. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. *Arch. Environ. Contam. Toxicol.* **22**(3), 278-283 (1992).

31. Mommsen, T. P., Vijayan, M. M. & Moon, T. W. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic re. *Rev. Fish Biol. Fisheries* **9**, 211–268, https://doi:10.1023/A:1008924418720 (1999).

32. Auperin, B. & Geslin, M. Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. *Gen. Comp. Endocrinol.* **158**(3), 234-239, https://doi:10.1016/j.ygcen.2008.07.002 (2008).

33. Steenbergen, P. J., Richardson, M. K. & Champagne, D. L. The use of the zebrafish model in stress research. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **35**(6), 1432-1451, https://doi: 10.1016/j.pnpbp.2010.10.010 (2011).

34. Hartig, E. I., Zhu, S., King, B. L. & Coffman, J. A. Cortisol-treated zebrafish embryos develop into pro-inflammatory adults with aberrant immune gene regulation. *Biol. Open* **5**(8), 1134-1141, https://doi:10.1242/bio.020065 (2016).

35. Wilson, K. S., Tucker, C. S., Al-Dujaili, E. A., Holmes, M. C., Hadoke, P. W., Kenyon, C. J. & Denvir, M. A. Early-life glucocorticoids programme behaviour and metabolism in adulthood in zebrafish. *J. Endocrinol.* **230**(1), 125-142, https://doi:10.1530/joe-15-0376 (2016).

36. Fries, E., Hesse, J., Hellhammer, J. & Hellhammer, D. H. A new view on hypocortisolism. *Psychoneuroendocrinolo.* **30**(10), 1010-1016, https://doi:10.1016/j.psyneuen.2005.04.006 (2005).

37. Heim, C., Ehlert, U. & Hellhammer, D. H. The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. *Psychoneuroendocrinol*. **25**(1), 1-35 (2000).

38. Hellhammer, D. H. & Wade, S. Endocrine correlates of stress vulnerability. *Psychother. Psychosom.* **60**(1), 8-17 (1993).

39. Rosenblueth, A., Wiener, N. & Bigelow, J. Behavior, Purpose and Teleology. *Philosophy of Sci.* **10**, 18-24 (1943).

40. Bagatto, B., Pelster, B. & Burggren, W. W. Growth and metabolism of larval zebrafish: effects of swim training. *J. Exp. Biol.* **204**, 4335-4343 (2001).

41. Santarelli, S., Lesuis, S. L., Wang, X. D., Wagner, K. V., Hartmann, J., Labermaier, C., Scharf, S. H., Muller, M. B., Holsboer, F. & Schmidt, M. V. Evidence supporting the match/mismatch hypothesis of psychiatric disorders. *Eur. Neuropsychopharmacol.* **24**, 907-918 (2014).

42. Zalosnik, M. I., Pollano, A., Trujillo, V., Suarez, M. M. & Durando, P. E. Effect of maternal separation and chronic stress on hippocampal-dependent memory in young adult rats: evidence for the match-mismatch hypothesis. *Stress* **11**, 1-6 (2014).

43. Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M. & Meaney, M. J. Epigenetic programming by maternal behavior. *Nat. Neurosci.* **7**, 847-854 (2004).

44. Westerfield, M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish* (*Danio rerio*) 4th edn (University of Oregon Press, 2000).

45. Yeh, C. M., Glöck, M. & Ryu, S. An optimized whole-body cortisol quantification method for assessing stress levels in larval zebrafish. *PLoS ONE* **8**, https://doi:10.1371/journal.pone.0079406 (2013).

46. Ryu, S. & De Marco, R. J. Performance on innate behaviour during early development as a function of stress level. *Scientific Reports* **7**, 7840, https://doi:10.1038/s41598-017-08400-4 (2017).

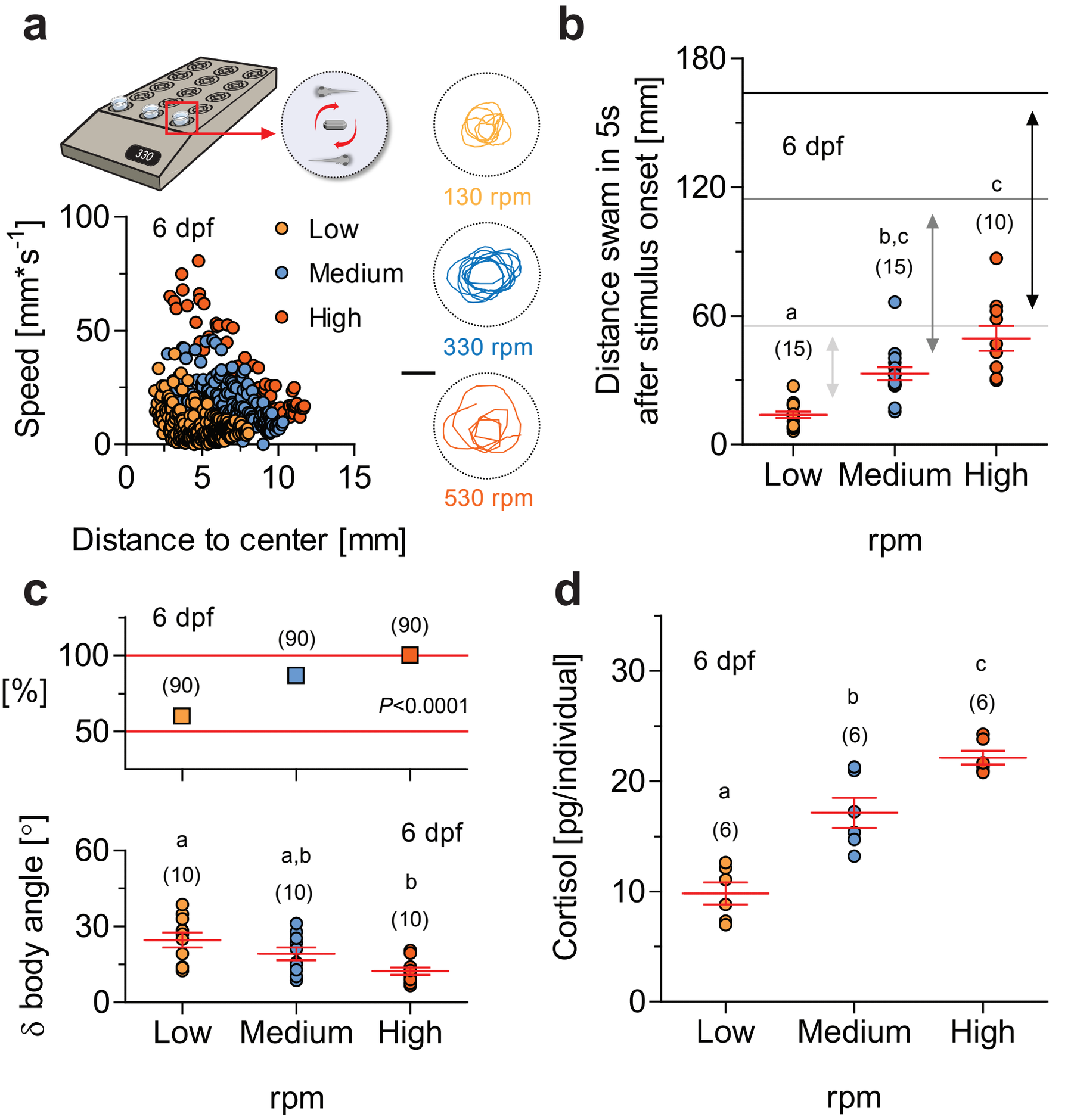
Figure Legends

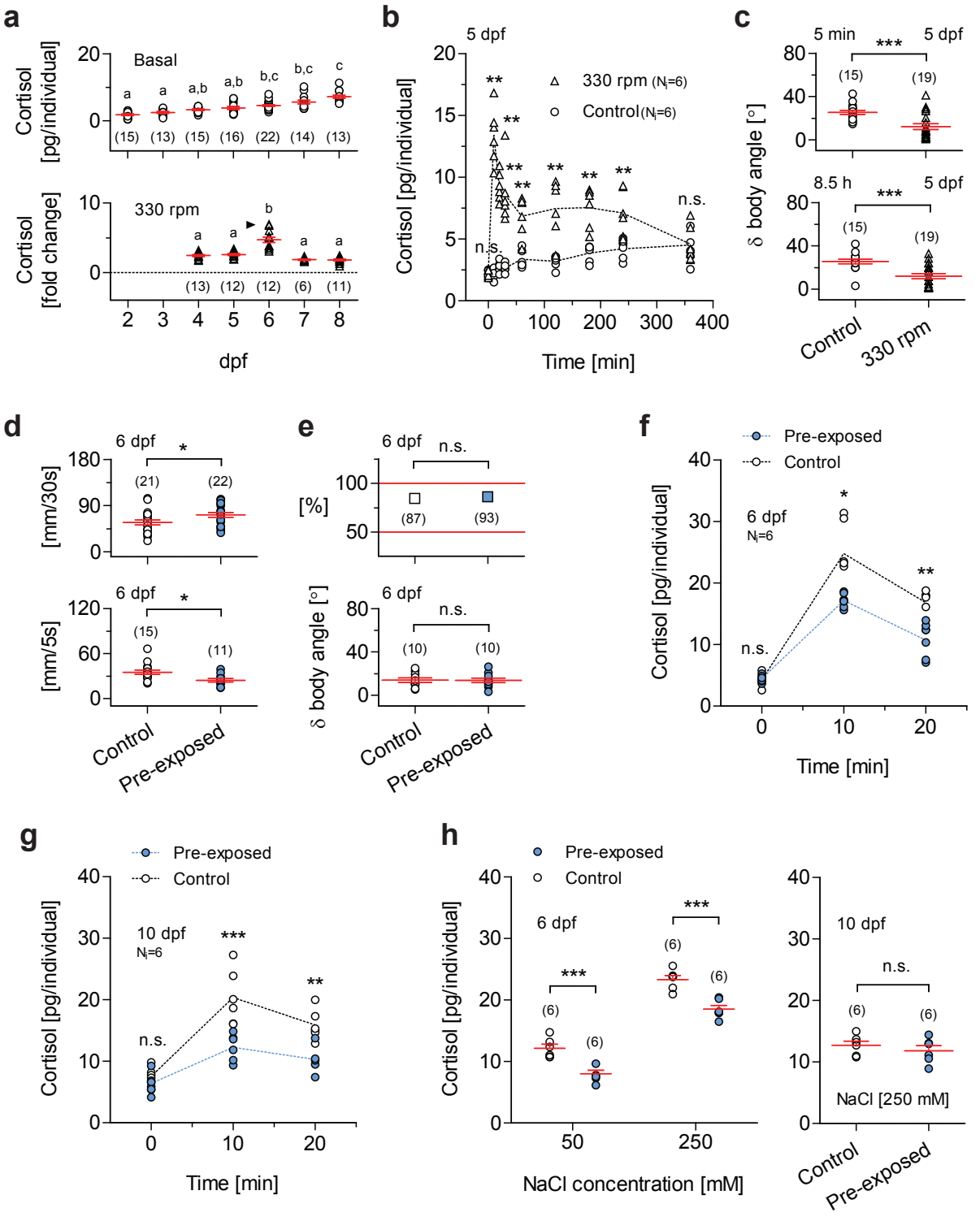
Figure 1. High-throughput induction of forced swimming and cortisol increase. (**a**) Top, schematic showing how groups of thirty zebrafish larvae in 35 mm diameter petri dishes can be exposed to water vortex flows in a high-throughput manner while their behaviour is being video-recorded (see the 'Methods' section). Right, representative x-y coordinates (recorded every 93.3 ms over a 2.4 s period) of single anesthetized 6 dpf larvae exposed to vortex flows of increasing strength, expressed in revolutions per minute (rpm). Scale bar, 10 mm. Bottom, swim velocity and distance to the center of the dish of a larva exposed to vortex flows of low (orange), medium (blue) and high (vermilion) strength levels (data from the top figures). (**b**) Distance swam in 5 s by freely behaving larvae after the onset of vortex flows as a function of vortex strength (as in **a**). Grey and black lines indicate the average distance covered by anesthetized larvae under similar conditions; double headed arrows highlight the differences between anesthetized and freely behaving larvae due to rheotaxis: the higher the vortex strength the lower the distance covered by individuals engaged in rheotaxis. Letters indicate results of Dunn's multiple comparison tests ($p < 0.01$) after a Kruskal-Wallis test. (**c**) Top, Proportion of larvae engaged in rheotaxis (measured 120 s after the onset of vortex flows) as a function of vortex strength (as in **a**); $P<0.0001$ after a Chi-square test. Bottom, Average change in orientation after a swim bout (δ body angle, in degrees, recorded every 933 ms over a 5 s period 120 s after the onset of vortex flows) of freely swimming larvae as a function of

vortex strength (as in **a**). (**d**) Whole-body cortisol in 6 dpf larvae as a function of vortex strength (as in **a**). (**c**,**d**) Letters indicate results of Bonferroni's tests ($p < 0.01$) after one-way ANOVAs. (**b,c**,**d**) Sample size in parentheses.

Figure 2. Prolonged forced swimming during early development increases spontaneous activity and reduces startle and glucocorticoid reactivity. (**a**) Top, Basal cortisol as a function of time, expressed in days post fertilization (dpf). Bottom, Cortisol change in response to vortex flows of medium strength (330 rpm) as a function of development, in dpf. Letters indicate results of Dunn's multiple comparison tests ($p < 0.01$) after Kruskal-Wallis tests. (**b**) Cortisol time course in 5 dpf larvae exposed to vortex flows (330 rpm) for 9 hours (shown up to 6 hours) and controls (unexposed larvae). Cortisol in exposed larvae peaks shortly after the onset of the vortex and remains high 4 hours later; exposed and control larvae show similar values 6 hours after the onset of the vortex. (**c**) δ body angle (as in **Fig. 1c**), indicative of rheotaxis, in exposed and control 5 dpf larvae, measured 5 minutes (top) and 8.5 hours (bottom) after the onset of the vortex (330 rpm). $^{**}P<0.01$, $^{***}P<0.001$ after two-tailed *t*-tests. (**d**) Top, spontaneous activity (in mm swam in 30s) in pre-exposed and control 6 dpf larvae. Bottom, locomotor reaction to the onset of the vortex (330 rpm) (mm swam in 5s, measured 5 s after the onset) in pre-exposed and control 6 dpf larvae. Pre-exposed larvae, blue. Control larvae, white. $P=0.03$ (top) and $P=0.01$ (bottom) after two-tailed *t*-tests. (**e**) Proportion of individuals engaged in rheotaxis (top) and δ body angle (bottom) (as in **Fig. 1c**) in pre-exposed (blue) and control (white) 6 dpf larvae. Top, $P=0.24$ after a Chi-square test. Bottom, $P=0.97$ after a two-tailed *t*-test. (**f,g**) Cortisol in pre-exposed (blue) and control (white) 6 dpf (**f**) and 10 dpf (**g**) larvae, before, 10 and 20 minutes after the onset of the vortex. (**h**) Cortisol in pre-exposed (blue) and control (white) 6 dpf (left) and 10 dpf (right) larvae in response to a 10 min incubation in hyperosmotic medium. Right, $P=0.42$ after a two-tailed *t*-test. (**b,f**) Asterisks ($^{*}P<0.05$, $^{**}P<0.01$) indicate results of two-group comparisons using

Mann-Whitney or two-tailed *t*-tests after Kruskal-Wallis tests. (**g,h**) Asterisks (**$P<0.01$, ***$P<0.001$) indicate results of Bonferroni's tests after two-way ANOVAs. (**b,f,g**) Sample size per group, 6. (**a,c,d,e,h**) Sample size in parentheses.


a
330
130 rpm
330 rpm
530 rpm
6 dpf
Low
Medium
High
Speed [mm*s-1]
Distance to center [mm]
b
6 dpf
Distance swam in 5s after stimulus onset [mm]
a
(15)
b,c
(15)
c
(10)
Low
Medium
High
rpm
c
6 dpf
[%]
(90)
(90)
(90)
P<0.0001
6 dpf
δ body angle [°]
a
(10)
a,b
(10)
b
(10)
Low
Medium
High
rpm
d
6 dpf
Cortisol [pg/individual]
a
(6)
b
(6)
c
(6)
Low
Medium
High
rpm


a
Basal
Cortisol [pg/individual]
a
a
a,b
a,b
b,c
b,c
c
(15) (13) (15) (16) (22) (14) (13)
330 rpm
Cortisol [fold change]
a
a
b
a
a
(13) (12) (12) (6) (11)
dpf
b
5 dpf
330 rpm (Ni=6)
Control (Ni=6)
Cortisol [pg/individual]
n.s.
n.s.
Time [min]
c
5 min
5 dpf
***
(15)
(19)
8.5 h
5 dpf
***
(15)
(19)
δ body angle [°]
Control
330 rpm
d
6 dpf
*
(21)
(22)
[mm/30s]
6 dpf
*
(15)
(11)
[mm/5s]
Control
Pre-exposed
e
6 dpf
n.s.
[%]
(87)
(93)
6 dpf
n.s.
(10)
(10)
δ body angle [°]
Control
Pre-exposed
f
Pre-exposed
Control
6 dpf
Ni=6
Cortisol [pg/individual]
n.s.
Time [min]
g
Pre-exposed
Control
10 dpf
Ni=6
Cortisol [pg/individual]
n.s.
Time [min]
h
Pre-exposed
Control
6 dpf
***
(6)
(6)
***
(6)
(6)
Cortisol [pg/individual]
NaCl concentration [mM]
10 dpf
n.s.
(6)
(6)
NaCl [250 mM]
Control
Pre-exposed