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1 **Active behaviour during early development shapes glucocorticoid reactivity**

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3 Luis A. Castillo-Ramírez[1], Soojin Ryu[1*], Rodrigo J. De Marco[1,2*]

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5 [1]Developmental Neurobiology of Resilience, German Resilience Center, University Medical

6 Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, 55128 Mainz, Germany

7 [2]Max Planck Institute for Medical Research, Jahnstr. 29, 69120 Heidelberg, Germany

8

9 [*]**Authors for correspondence**:

10 E-mail: R.J.D.M. <rjdm@protonmail.com>, S.R. <soojin.ryu@uni-mainz.de>

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12 **Abstract**

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

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22 **Introduction**

23 The increased secretion of glucocorticoids like cortisol after the onset of stress (a.k.a.)
24 glucocorticoid reactivity (GC_R) plays a pivotal role in the response to challenge. It is critical
25 for adaptation and central to an organism's resilience (Sapolsky et al., 2000). GC_R is a tightly
26 regulated phenomenon, a response of the hypothalamic-pituitary-adrenal (HPA) axis to
27 exogenous or endogenous stressors. Altered functionality of the HPA axis and of GC_R have
28 been associated with detrimental and beneficial consequences for health. They have been
29 linked to stress-evoked disorders including mental disorders as well as increased resilience
30 (Daskalakis et al., 2013; Khulan and Drake, 2012; McEwen, 2008; Nederhof and Schmidt,
31 2012; Reynolds, 2013; Schmidt, 2010; Seckl and Meaney, 2004). Glucocorticoid secretion
32 has been investigated extensively under steady-state and stress conditions (Reul et al., 2014),
33 and there is ample evidence that HPA axis functionality is susceptible to disturbance by early
34 life stress. Early adversity can, for example, alter glucocorticoid regulation and coping
35 capacities later in life (Russo et al., 2012; Strüber et al., 2014). However, it is still unclear
36 how active responses to early life stress can reconfigure HPA axis function, pending a
37 detailed functional evaluation of developmental programming of GC_R. Larval zebrafish are
38 excellent to address this knowledge gap due to their external development, their
39 hypothalamic-pituitary-interrenal (HPI) axis, homologous to the mammalian HPA axis
40 (Wenderlaar Bonga, 1997), their translucent body, ideal for non-invasive brain imaging and
41 optogenetics (De Marco et al., 2016; Gahtan and Baier, 2004; Portugues et al., 2013), their
42 small size, highly suitable for high-throughput screens with full environmental control, and
43 the availability of tools and methods for identifying genetic and epigenetic modulators,
44 including proteomic technology. Therefore, as a first step, we set out to determine the effect
45 of early life stress on GC_R and coping capacity in larval zebrafish. Taken together our results
46 introduce a high-throughput forced swim test for developing zebrafish and demonstrate that

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47 mild early life stress can at least transiently reconfigure GC_R and elicit modulatory
48 adjustments in spontaneous activity and startle reactivity.
49
50 **Results**
51 ***High-throughput induction of forced swimming and cortisol increase***
52 Firstly, we exposed groups of larvae to water vortex flows of varying strength, expressed in
53 revolutions per minute (rpm) (for details, see Methods). To compare the strength of these
54 flows, we video-recorded and examined the paths (x-y coordinates) of anesthetized larvae
55 (i.e., unable to swim) exposed to vortex flows of increasing rpm (Fig. 1a). The results of these
56 observations confirmed that, as rpm increased, anesthetized larvae followed the vortex
57 currents, thereby moving at higher speeds and larger distances from the source of the vortex.
58 These measurements were used to determine vortex flows of low, medium and high strength.
59 We then assessed the relationship between the strength of the flows and the behaviour of
60 freely swimming larvae. Larval zebrafish have been shown to display positive rheotaxis
61 (Olszewski et al., 2012; Suli et al., 2012), i.e., spontaneous swimming against an oncoming
62 current, which allows them to hold their position instead of being swept downstream by the
63 current. When exposed to vortex flows, freely swimming larvae held their position away from
64 the vortex's source, thereby avoiding the strongest currents (Fig. 1b, one-way ANOVA,
65 $F(2,39)=26.6$, $p < 0.0001$, followed by *post hoc* comparisons). They also faced the oncoming
66 current (Fig. 1c, top, Chi-square test, $X^2(1, N=270)=55.4$, $p < 0.0001$) and adjusted their swim
67 bouts and turns (Fig. 1c, bottom, one-way ANOVA, $F(2,29)=6.7$, $p = 0.004$, followed by *post*
68 *hoc* comparisons) to compensate for vortex strength. As indicated by their GC_R, these
69 behaviours were taxing for the larvae. Their whole-body cortisol increased together with the
70 strength of the vortex (Fig. 1d, one-way ANOVA, $F(2,17)=35.4$, $p < 0.0001$, followed by *post*
71 *hoc* comparisons).
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73 ***Cortisol change in response to water vortex flows as a function of development***

74 Secondly, we assessed GC_R to vortex flows as a function of development, expressed in days
75 post fertilization (dpf). For these tests we selected vortex flows of medium strength (i.e., 330
76 rpm), avoiding the high strength causing maximum levels of vortex-dependent cortisol
77 increase and occasional disruptions of positive rheotaxis (not shown). The HPI axis of
78 zebrafish matures early. Basal whole-body cortisol and expression levels of genes involved in
79 corticosteroid synthesis and signaling increase drastically around the time of hatching (Alsop
80 and Vijayan, 2008; Alderman and Bernier, 2009). We observed that whole-body cortisol
81 increased gradually between 2 and 8 dpf (Fig. 2a, top, one-way ANOVA, $F(6,107)=23.0$, $p <$
82 0.0001, followed by *post hoc* comparisons), and that the magnitude of the vortex-dependent
83 elevation of cortisol peaked at 6 dpf (Fig. 2a, bottom, one-way ANOVA, $F(4,53)=27.2$, $p <$
84 0.0001, followed by *post hoc* comparisons), with circulating levels of cortisol measured ten
85 minutes after a three minute exposure to vortex flows (for details, see Methods).
86 Mechanistically, this points to fundamental alterations in the HPI axis occurring at 4-6 dpf.
87

88 ***Prolonged forced swimming and HPI activation at 5 dpf***

89 Thirdly, building on the above findings, we exposed 5 dpf larvae to vortex flows of medium
90 strength for 9 hours. Exposed larvae showed increased levels of whole-body cortisol which
91 peaked shortly after the onset of the vortex and remained high four hours later compared with
92 controls, i.e., unexposed larvae that were equally handled, but the vortex flows were not
93 present. Both exposed and control larvae showed similar levels of whole-body cortisol six
94 hours after the onset of the vortex (Fig. 2b, two-way ANOVA, group: $F(1,90)=263.3$, $p <$
95 0.0001, time: $F(8,90)=13.8$, $p < 0.0001$, group x time: $F(8,90)=19.4$, $p < 0.0001$, followed by
96 *post hoc* comparisons). Also, exposed larvae remained engaged in positive rheotaxis from the
97 beginning to the end of the vortex, as indicated by their δ body angle (average change in
98 orientation after a swim bout) measured 5 minutes (Fig. 2c, top, two-tailed *t*-test, $t(32)=3.8$, P

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99 = 0.0007) and 8.5 hours (Fig. 2c, bottom, two-tailed *t*-test, t(32)=4.1, $P = 0.0003$) after the
100 onset of vortex flows (see also Methods).
101
102 ***Increased baseline swimming and reduced startle reactivity in pre-exposed larvae***
103 Next, using video-recordings and off-line measurements (for details, see Methods), we
104 assessed the behaviour of 6 dpf larvae that had or had not been exposed to vortex flows at 5
105 dpf, i.e., pre-exposed and control larvae, respectively. Compared with controls, pre-exposed
106 larvae showed higher levels of baseline swimming (Fig. 2d, top, two-tailed *t*-test, t(41)=2.2, *P*
107 = 0.03) and reduced startle reactivity upon re-exposure to vortex flows of medium strength, as
108 specified by the distance they swam directly after the onset of the water current (Fig. 2d,
109 bottom, two-tailed *t*-test, t(24)=2.7, $P = 0.01$). Importantly, pre-exposed larvae engaged in
110 positive rheotaxis as efficiently as controls, as indicated by the proportion of larvae facing the
111 oncoming current (Fig. 2e, top, Chi-square test, X^2(1, N=180)=1.4, $p = 0.24$) and δ body
112 angle (Fig. 2e, bottom, two-tailed *t*-test, t(18)=0.08, $P = 0.94$).
113
114 ***Reduced glucocorticoid reactivity to vortex flows in pre-exposed larvae***
115 At 6 dpf, pre-exposed larvae, which had prior experience with the vortex flows at 5 dpf,
116 showed reduced GC_R as well as the above behavioural adjustments. Relative to controls, pre-
117 exposed larvae showed similar levels of basal cortisol and reduced levels of vortex-dependent
118 cortisol increase upon re-exposure to vortex flows of medium strength (Fig. 2f, two-way
119 ANOVA, group: F(1,30)=24.2, $p < 0.0001$, time: F(2,30)=111.1, $p < 0.0001$, group x time:
120 F(2,30)=6.9, $p = 0.003$, followed by *post hoc* comparisons). We found the same pattern of
121 results at 10 dpf (Fig. 2g, two-way ANOVA, group: F(1,30)=32.8, $p < 0.0001$, time:
122 F(2,30)=40.5, $p < 0.0001$, group x time: F(2,30)=5.6, $p = 0.009$, followed by *post hoc*
123 comparisons).

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124

125 ***Short-term reduced glucocorticoid reactivity to heterotypic stress***

126 To complement these assessments we examined the relationship between GC_R in pre-exposed
127 larvae and heterotypic stress. For this we exposed 6 dpf pre-exposed and control larvae to
128 hyperosmotic medium (NaCl), a known stress protocol (for details, see Methods). The results
129 showed that, relative to controls, pre-exposed larvae showed reduced GC_R to moderate and
130 high levels of salt stress (Fig. 2h, left, two-way ANOVA, group: $F(1,20)=51.0$, $p < 0.0001$,
131 NaCl concentration: $F(1,20)=299.0$, $p < 0.0001$, group x NaCl concentration: $F(1,20)=0.3$, $p =$
132 0.59, followed by *post hoc* comparisons). By contrast, at 10 dpf, both groups showed similar
133 cortisol responses to salt stress (Fig. 2h, right, two-tailed *t*-test, $t(10)=0.9$, $P = 0.42$).

134

135 **Discussion**

136 Hormones react to the environment and cause changes in physiology as a function of
137 maturation. Thus the question arises as to how dynamical patterns of hormone secretion are
138 achieved and what effects they exert on well-being. How does the environment activate and
139 guide the development of resilience mechanisms? Current paradigms stipulate that
140 glucocorticoids are fundamental to the mitigation of allostatic load (Reul et al., 2014;
141 McEwen, 2001). However, the impact of early life stress on developmental programming of
142 GC_R has not been explored in full, in part due to a lack of suitable models. We now show in
143 zebrafish that the increase in cortisol elicited by a brief period of involuntary swimming peaks
144 at 6 days post fertilization (dpf), pointing to fundamental changes in GC_R at early larval
145 stages. Importantly, we found that, if prolonged for hours, forced swimming at 5 dpf caused a
146 transient form of hypercortisolaemia and later led to reduced GC_R. If subsequently exposed to
147 a brief period of involuntary swimming (i.e., homotypic stress), pre-exposed larvae showed a
148 decreased cortisol response that persisted for at least five more days. Moreover, twenty-four

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149 hours after prolonged forced swimming at 5 dpf, the reduced GC_R appeared invariant to
150 stressor identity, as indicated by a decreased cortisol response to heterotypic stress, i.e.,
151 osmotic shock. These data suggested that the sustained reduction in GC_R did not reflect a
152 process of habituation to sensory input (Grissom and Bhatnagar, 2009). It seems likely that, in
153 pre-exposed larvae, reduced GC_R was underpinned by changes in state variables of the HPI
154 axis.

155 Early adversity and chronic stress later in life can decrease hypothalamic activity and
156 expression of corticotropin-releasing-hormone (CRH) and arginine-vasopressin (AVP). These
157 changes are mediated at least in part by glucocorticoids (Erkut et al., 1998; Herman et al.,
158 2008; Tasker and Herman, 2011; Wismer Fries et al., 2005). Previous studies in fish showed
159 that prolonged stimulation of the HPI axis can attenuate the stress response (Barton, 2002).
160 This effect can result from transcriptional regulation of CRH and adrenocorticotropic
161 hormone (ACTH) (Birnberg et al., 1983; Eberwine and Roberts, 1984; Imaki et al., 1991).
162 Additionally, pituitary corticotrophs and cortisol-producing cells in the interrenal gland may
163 be desensitized to CRH or ACTH, respectively (Hontela et al., 1992; Mommsen, 1999). It has
164 been shown in rainbow trout that stressor exposure at early developmental stages can lead to
165 HPI axis hypoactivity later in life (Auperin and Geslin, 2008). In zebrafish, incubation in
166 cortisol during the first 48 hours post fertilization caused altered locomotor reactions to photic
167 stimuli (Steenbergen et al., 2011). Also, cortisol incubation of zebrafish embryos during the
168 first five days post fertilization increased whole-body cortisol, glucocorticoid signalling and
169 expression of immune system-related genes; these changes can be long-lasting and result in
170 dysfunctional regeneration capacities and increased expression of inflammatory genes (Hartig
171 et al, 2016). A similar treatment using dexamethasone also induced long-lasting behavioural
172 and metabolic changes still detectable in adulthood (Wilson et al., 2016). Further experiments
173 are necessary to determine whether reduced GC_R in pre-exposed larvae occurs via receptor
174 downregulation, decreased synthesis and/or depletion of hormones, and/or increased

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175 sensitivity to glucocorticoid feedbacks (Fries et al., 2005; Heim et al., 2000; Hellhammer and
176 Wade, 1993).

177 An organism is said to be engaged in active behaviour when it is the source of the
178 output energy required for a given action (Rosenblueth et al., 1943). Glucocorticoids are
179 known to mobilize energy (Sapolsky et al., 2000), which is necessary to cope with the high
180 energy demands associated with forced swimming. In response to the vortex, larvae engaged
181 in rheotaxis had to adjust their swim bouts and turns continuously to compensate for the
182 oncoming current. These actions were energy demanding for the larvae, as revealed by their
183 GC_R. The notion that upholding positive rheotaxis for hours involved mobilizing energy was
184 supported by the long-lasting hypercortisolic state observed during prolonged forced
185 swimming. Twenty-four hours after prolonged exposure to the vortex, we observed
186 differences between pre-exposed and control larvae. Firstly, pre-exposed larvae displayed
187 increased levels of baseline swimming. A previous study in zebrafish showed that involuntary
188 swimming at larval stages can subsequently increase spontaneous activity (Bagatto et al.,
189 2001). Secondly, upon a brief re-exposure to the same vortex, pre-exposed larvae showed
190 reduced startle reactivity to the onset of water motions. Thirdly, they engaged in positive
191 rheotaxis as efficiently as controls. On the assumption that the cortisol response to the vortex
192 reflects an energy requirement for positive rheotaxis, these observations indicated that pre-
193 exposed larvae responded more efficiently to the energy demands of forced swimming.

194 In conclusion, we have shown in larval zebrafish that early life stress caused by
195 prolonged forced swimming at least transiently reconfigures the increased secretion of
196 cortisol after the onset of homotypic or heterotypic stress, as well as spontaneous activity and
197 efficient energy use during active behaviour. It remains open how these changes relate to
198 survival in a species facing greater mortality during early life; there is a lack of evidence
199 linking early activity patterns of the HPI axis to survival and reproductive outcome.
200 Collectively, our data provided direct evidence to support the contention that long-term

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201 changes in HPI axis function after early adversity may later lead to increased resilience in
202 developing zebrafish. Increased resilience to stress may have advantages for larval zebrafish.
203 Such ability may help larvae to better cope with antagonistic environments. An important
204 question emerging relates to the study of stress reactivity during adulthood as a function of
205 early life events in zebrafish. A previous study in mice reported that individuals that had
206 endured early life stress coped better with forced swimming compared with those that had
207 experienced a favourable early care regime (Santarelli et al., 2014). In adult rats, the adverse
208 experience of maternal separation during early life strengthened freezing during fear
209 conditioning after chronic stress compared with non-maternally separated rats (Zalosnik et al.,
210 2014). These studies support the view that early life stress can lead to increased resilience in
211 later life. Long-lasting changes in HPA axis function due to early experiences have been
212 attributed to changes in the epigenome (Weaver et al., 2004). However, the link between early
213 life stress and the activation of resilience mechanisms has been difficult to pin down in
214 models with intrauterine development. In zebrafish, all three elements of the HPI axis can be
215 visualized and genetically manipulated at early developmental stages and measured with
216 modern molecular tools (De Marco et al., 2016). Moreover, the larval brain is readily
217 accessible and provide excellent access for assessing how systematic variations in
218 physiological and behavioural schemes relate to differences in the activity of neuronal and
219 humoral networks. Further studies are required to determine the applicability of our high-
220 throughput procedure. In anticipation to these studies, we speculate that zebrafish larvae will
221 prove fruitful to link early HPI axis activity to proteomic regulation, epigenetic programming
222 and measures of stress resilience.

223

224 Methods

225 *Zebrafish husbandry and handling*

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226 Zebrafish breeding and maintenance were performed under standard conditions (Westerfield,
227 2000). Groups of thirty wild-type embryos (cross of AB and TL strains, AB/TL) were
228 collected in the morning and raised on a 12:12 light/dark cycle at 28 °C in 35 mm Petri dishes
229 with 5 ml of E2 medium. At 3 days post fertilization (dpf), the E2 medium was renewed and
230 chorions and debris were removed from the dishes. Experiments were carried out with 5-6 dpf
231 larvae, with the exception of the cortisol measurements in Fig. 2a, g and h(left). Larvae older
232 than 6 dpf were transferred to plastic cages with 400 ml of egg water in groups of thirty and
233 fed with paramecia daily. Tests were performed between 09:00 hours and 18:00 hours, with
234 different experimental groups intermixed throughout the day. Zebrafish experimental
235 procedures were performed according to the guidelines of the German animal welfare law and
236 approved by the local government (Regierungspräsidium Karlsruhe; G-29/12).

237 *Water vortex flows*

238 Water current can trigger rheotaxis in larval zebrafish and, if sufficiently strong, it can also act
239 as a stressor, causing a sharp increase in whole-body cortisol via the activation of the HPI
240 axis. We used water vortex flows in a high-throughput fashion to induce both rheotaxis and
241 cortisol increase. For this we exposed groups of thirty (4-8 dpf) larvae in 35 mm Petri dishes
242 with 5 ml of E2 medium to the vortex flows caused by the spinning movements of small
243 magnetic stir bars (6 x 3mm, Fischerbrand, #11888882, Fisher scientific, Leicestershire, UK.)
244 inside the dishes. The Petri dishes, each with a single stir bar, were positioned on magnetic
245 stirrer plates (Variomag, Poly 15; Thermo Fisher Scientific, Leicestershire, UK) and kept at
246 28°C inside an incubator (RuMed 3101, Rubarth Apparate GmbH, Laatzen, Germany).
247 Larvae were presented with either short (3 minutes) or long (9 hours of continuous
248 stimulation) exposure periods to the vortex flows caused by the highly-controlled magnetic
249 field inversions of the stirrer plate, of 130, 330 or 530 revolutions per minute (rpm). For the
250 short exposure, we avoided exposure periods longer than 3 minutes to elude maximum levels
251 of stressor-mediated cortisol increase (not shown). The long exposure at 5 dpf consisted of 9

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252 hours to achieve the longest possible exposure period adjustable to the light/dark cycle. Once
253 exposed, larvae were immobilized in ice water and used for cortisol measurement (see below).
254 Control larvae were collected after equal handling, omitting exposure to vortex flows (i.e., stir
255 bars inside the Petri dishes were absent). To rule out unspecific effects of the magnetic field
256 inversions produced by a stirrer plate, we compared the level of basal whole-body cortisol
257 across groups of 6 dpf larvae that either remained unexposed or had been exposed to magnetic
258 field inversions alone (i.e., without stir bars inside the Petri dishes and thus in the absence of
259 vortex flows), of 130, 330 and 530 rpm. The results of these tests showed that magnetic field
260 inversions per se did not alter the level of whole-body cortisol (one-way ANOVA,
261 $F(3,23) = 0.05$, $p = 0.98$).

262 *Re-exposure to vortex flows*

263 For these tests we selected vortex flows of medium strength to avoid possible ceiling effects
264 caused by maximum levels of vortex-dependent cortisol increase. Using the above protocol,
265 larvae that had or had not been exposed to vortex flows for 9 hours at 5 dpf were re-exposed
266 to vortex flows (330 rpm) for 3 minutes at either 6 or 10 dpf. They were subsequently used
267 for cortisol detection or behaviour evaluation.

268 *Re-exposure to vortex flows at 10 dpf*

269 A plastic cage (5 L) containing thirty pre-exposed or control 10 dpf larvae and three magnetic
270 stir bars (25 x 6 mm, Fisherbrand, #10226853, Fisher scientific, Leicestershire, UK)
271 distributed equidistantly along the bottom of the cage were placed on top of the magnetic
272 stirrer plate (Variomag, Poly 15, Thermo Scientific, Leicestershire, UK). Larvae were then
273 exposed to vortex flows (330 rpm) for 3 minutes. Larvae were then immobilized with ice
274 water and used for cortisol extraction 10 minutes after the onset of the vortex flows.

275 *Hyperosmotic medium*

276 Groups of thirty larvae (either 6 or 10 dpf) in 35 mm Petri dishes were incubated for 10 min in
277 steady state E2 medium (controls) or E2 + 50 or 250 mM NaCl (Merck, #106404, Darmstadt,

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278 Germany) at 28°C under white light illumination. They were washed three times with E2
279 medium and kept for immediate cortisol detection. The wash and transfer period took 3 min
280 (± 10 s) and was performed at room temperature.

281 *Whole-body cortisol*

282 Groups of thirty larvae were immobilized in ice water after being exposed to water vortex
283 flows or NaCl. Unexposed larvae (control samples) were collected after equal handling,
284 omitting stressor exposure. Samples were then frozen in an ethanol/dry-ice bath and stored at
285 −20 °C for subsequent extraction. Each replicate consisted of a well with 30 larvae. Cortisol
286 extraction and detection were carried between 10:30 and 11:30 hours out using a home-made
287 cortisol ELISA protocol, as described elsewhere (Yeh et al., 2013).

288 *Independent sampling*

289 Cortisol and behavioural measurements were made on different groups of equally treated
290 larvae and therefore constitute fully independent samples. For the behavioural measurements,
291 each replicate involved a single larva. Yet, these individual measurements were made on
292 larvae that had also been kept in wells containing a total of thirty larvae per well. Thus, the
293 number of single larvae matched the number of independent wells. In this manner, the density
294 of larvae per well during vortex flow exposure remained a constant factor for both the cortisol
295 and behavioural measurements. For each cortisol measurement, all thirty larvae in a well were
296 used, whereas each behavioural measurement involved only one larva, the remaining twenty-
297 nine larvae in the well were used elsewhere. Each replication was fully independent from the
298 others thus avoiding pseudo-replication.

299 *Anesthetized larvae*

300 To assess the speed and trajectories of anesthetized larvae exposed to vortex flows of
301 increasing strength, 6 dpf larvae in 35 mm Petri dishes were first incubated in 5 mL of steady
302 state E2 medium + 100 µL of Tricaine (Sigma-Aldrich #E10521, Schnelldorf, Germany); they

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303 were considered to be anesthetized when they failed to respond to tactile stimulation. They
304 were then transferred to a new Petri dish with fresh E2 medium (5 mL) for testing.
305 *Behaviour evaluation*
306 Video recordings were conducted under conditions identical to those of the cortisol
307 measurements. Groups of thirty larvae (either 5 or 6 dpf, depending on the experiment) were
308 imaged at 12.5 frames s^{-1} with a camera (HDR-CX240 HD Flash, Sony, Berlin, Germany)
309 positioned above a 35 mm Petri dish with 5 ml of E2 medium and a magnetic stir bar placed
310 on a magnetic stirrer plate inside the incubator, as described above. Videos samples were later
311 used for offline data recovery using ImageJ 1.48v software (National Institutes of Health,
312 Bethesda, USA) and MTrackJ (Biomedical Imaging Group Rotterdam, Rotterdam, The
313 Netherlands). Larvae were individually tracked and their x-y coordinates at every time point
314 were subsequently used to calculate motion values, body orientation and position relative to
315 the rotation axis of the magnetic stir bar, which corresponded in all cases to the center of the
316 Petri dish. Motion values were expressed as either speed (mm per second) or distance swum
317 every 5 or 30 seconds. To quantify the proportion of larvae engaged in rheotaxis, we
318 measured the proportion of larvae directly facing the oncoming current 120 s after the onset
319 of vortex flows. For this we measured - three times every 10 s - the angle formed between a
320 larva's body axis and a line connecting the center of its head and the rotation axis of the
321 magnetic stir bar. A larva was considered to be engaged in rheotaxis when the coefficient of
322 variation arising from the three angles measured over 30 s remained lower than 10 % and, at
323 the same time, it exhibited minimum body displacements, i.e., shorter than 0.5 mm * (10
324 $ms)^{-1}$. To assess the average change in orientation after a swim bout (δ body angle, in
325 degrees), we measured, as before, the angle formed between a larva's body axis and a line
326 connecting the center of its head and the rotation axis of the magnetic stir bar, every 933 ms
327 over a 5 s period 120 s after the onset of vortex flows. The resulting 'δ body angle' values (in

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328 degrees) were then calculated as the average difference between the consecutive angles for
329 each larva.
330 *Statistics*
331 All data are shown as single measurement points or mean and standard error of the mean. We
332 used a random experimental design, Student's *t*-tests (two-tailed) for two-group comparisons,
333 Chi-square tests and ANOVAs for multiple group comparisons (followed by Bonferroni's
334 *post hoc* tests). Normality was tested using Kolmogorov–Smirnov, Shapiro–Wilk and
335 D'Agostino tests. Analyses were made with MS-Excel (Microsoft Corp; Redmond, WA,
336 USA), Prism 5 (Graphpad Software Inc, San Diego, CA, USA), ImageJ (Freeware) and
337 VirtualDub (Freeware).
338 *Data accessibility*
339 The data that support the findings of this study are available from the authors on request.
340
341 **Acknowledgments**
342 This work was supported by the Max Planck Society, the University Medical Center of the
343 Johannes Gutenberg University Mainz, the German Federal Office for Education and
344 Research (Bundesministerium für Bildung und Forschung, grant 01GQ1404) and the German
345 Research Foundation (DFG, grant A04- CRC 1193). We thank R. Singer and A. Schoell for
346 expert fish care.
347
348 **Author Contributions**
349 Conceptualization, R.J.D.M. and S.R.; Methodology, R.J.D.M. and S.R.; Investigation,
350 L.A.C-R., S.R. and R.J.D.M.; Writing – Original Draft, L.A.C-R and R.J.D.M.; Writing –
351 Review & Editing, R.J.D.M.
352
353 **Competing Interests**

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354 The authors declare that they have no competing interests.

355

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481

482 **Figure Legends**

483 **Figure 1. High-throughput induction of forced swimming and cortisol increase.** Groups
484 of thirty zebrafish larvae in 35 mm diameter petri dishes can be exposed to water vortex flows
485 in a high-throughput manner while their behaviour is being video-recorded. (**a**) Top,
486 representative x-y coordinates (recorded every 93.3 ms over a 2.4 s period) of single
487 anesthetized 6 dpf larvae exposed to vortex flows of increasing strength, expressed in
488 revolutions per minute (rpm). Scale bar, 10 mm. Bottom, swim velocity and distance to the
489 center of the dish of a larva exposed to vortex flows of low (orange), medium (blue) and high
490 (vermilion) strength levels (data from the top figures). (**b**) Distance swam in 5 s by freely
491 behaving larvae after the onset of vortex flows as a function of vortex strength (as in **a**). Grey
492 and black lines indicate the average distance covered by anesthetized larvae under similar
493 conditions; double headed arrows highlight the differences between anesthetized and freely
494 behaving larvae due to rheotaxis: the higher the vortex strength the lower the distance covered
495 by individuals engaged in rheotaxis. (**c**) Top, Proportion of larvae engaged in rheotaxis
496 (measured 120 s after the onset of vortex flows) as a function of vortex strength (as in **a**);
497 $P<0.0001$ after a Chi-square test. Bottom, Average change in orientation after a swim bout (δ
498 body angle, in degrees, recorded every 933 ms over a 5 s period 120 s after the onset of vortex
499 flows) of freely swimming larvae as a function of vortex strength (as in **a**). (**d**) Whole-body
500 cortisol in 6 dpf larvae as a function of vortex strength (as in **a**). (**b**,**c**,**d**) Letters indicate
501 results of Bonferroni's tests ($p < 0.01$) after one-way ANOVAs. Sample size in parentheses.

502

503 **Figure 2. Prolonged forced swimming during early development increases spontaneous**
504 **activity and reduces startle and glucocorticoid reactivity.** (**a**) Top, Basal cortisol as a
505 function of time, expressed in days post fertilization (dpf). Bottom, Cortisol change in
506 response to vortex flows of medium strength (330 rpm) as a function of development, in dpf.

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507 Letters indicate results of Bonferroni's tests ($p < 0.01$) after one-way ANOVAs. (**b**) Cortisol
508 time course in 5 dpf larvae exposed to vortex flows (330 rpm) for 9 hours (shown up to 6
509 hours) and controls (unexposed larvae). Cortisol in exposed larvae peaks shortly after the
510 onset of the vortex and remains high 4 hours later; exposed and control larvae show similar
511 values 6 hours after the onset of the vortex. (**c**) δ body angle (as in **Fig. 1c**), indicative of
512 rheotaxis, in exposed and control 5 dpf larvae, measured 5 minutes (top) and 8.5 hours
513 (bottom) after the onset of the vortex (330 rpm). **P<0.01, ***P<0.001 after two-tailed *t*-
514 tests. (**d**) Top, spontaneous activity (in mm swam in 30s) in pre-exposed and control 6 dpf
515 larvae. Bottom, locomotor reaction to the onset of the vortex (330 rpm) (mm swam in 5s,
516 measured 5 s after the onset) in pre-exposed and control 6 dpf larvae. Pre-exposed larvae,
517 blue. Control larvae, white. P=0.03 (top) and P=0.01 (bottom) after two-tailed *t*-tests. (**e**)
518 Proportion of individuals engaged in rheotaxis (top) and δ body angle (bottom) (as in **Fig. 1c**)
519 in pre-exposed (blue) and control (white) 6 dpf larvae. Top, P=0.24 after a Chi-square test.
520 Bottom, P=0.97 after a two-tailed *t*-test. (**f,g**) Cortisol in pre-exposed (blue) and control
521 (white) 6 dpf (**f**) and 10 dpf (**g**) larvae, before, 10 and 20 minutes after the onset of the vortex.
522 (**h**) Cortisol in pre-exposed (blue) and control (white) 6 dpf (left) and 10 dpf (right) larvae in
523 response to a 10 min incubation in hyperosmotic medium. Right, P=0.42 after a two-tailed *t*-
524 test. (**b,f,g,h**) Asterisks (**P<0.01, ***P<0.001) indicate results of Bonferroni's tests after
525 two-way ANOVAs. (**b,f,g**) Sample size per group, 6. (**a,c,d,e,h**) Sample size in parentheses.

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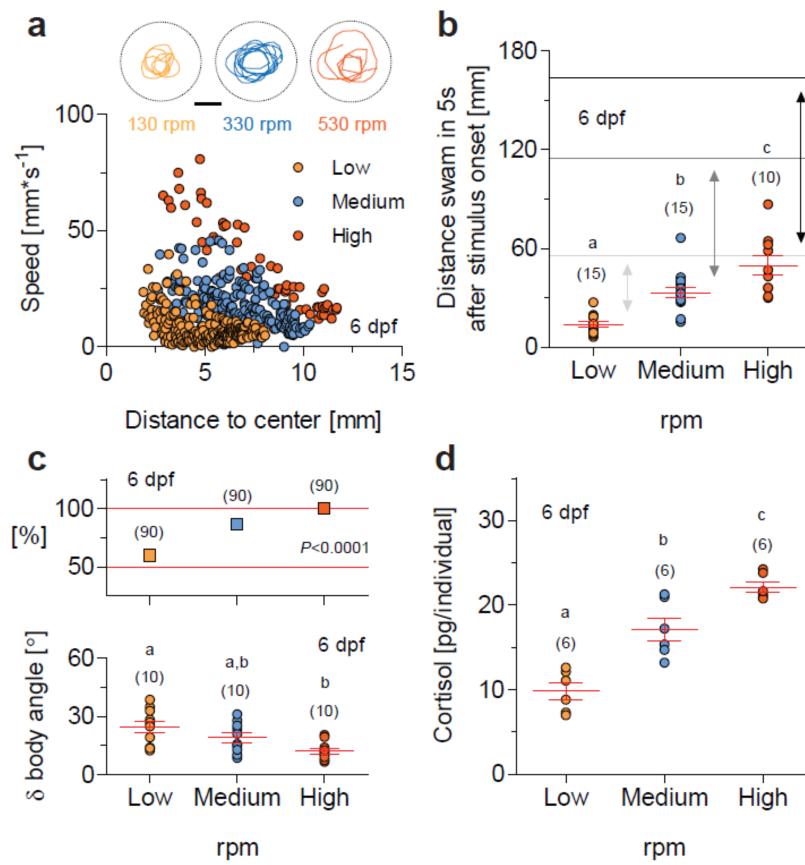
526 **Figure 1**



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527 **Figure 2**