Running title: Cortisol response in zebrafish larvae

Cortisol dynamics and GR-dependent feedback regulation in zebrafish larvae exposed to repeated stress

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Abstract. Zebrafish larvae show a rapid increase in cortisol in response to acute stressors, followed by a decline. While these responses are documented, both the duration of the refractory period to repeated stressors and the role of glucocorticoid receptors (GR) in specific phases of the glucocorticoid negative feedback are still being clarified. We explored these questions using water vortices as stressors, combined with GR blockage and measurements of whole-body cortisol in zebrafish larvae subjected to single and repeated stress protocols. Cortisol levels were elevated 10 minutes after stress onset and returned to baseline within 30-40 minutes, depending on the stressor strength. In response to homotypic stress, cortisol levels rose above baseline if the second stressor occurred 60 or 120 minutes after the first, but not with a 30-minute interval. This suggests a rapid cortisol-mediated feedback loop with a refractory period of at least 30 minutes. Treatment with a GR blocker delayed the return to baseline and suppressed the refractory period, indicating GR-dependent early-phase feedback regulation. These findings are consistent with mammalian models and provide a framework for further analyses of early-life cortisol responses and feedback in zebrafish larvae, ideal for non-invasive imaging and high-throughput screening.

Keywords: Cortisol response, Refractory period, Glucocorticoid negative feedback, GR antagonist, Zebrafish larvae

Introduction

Glucocorticoids (GCs) are released in response to stressors, helping the body cope with challenge (de Kloet et al., 1998; McEwen, 2007). They are regulated by the hypothalamic-pituitary-adrenal (HPA) axis in mammals and the hypothalamic-pituitary-interrenal (HPI) axis in fish. The HPA axis in mammals is homologous to the HPI axis in zebrafish, making zebrafish a valuable model for stress research (Alsop and Vijayan, 2008; Alsop and Vijayan, 2009; Egan et al., 2009; Champagne and Richardson, 2013; Biran et al., 2015; Eachus et al., 2021; Tan et al., 2022; Swaminathan et al., 2023; Herget et al., 2023). Both mammals and zebrafish process stress signals through analogous centres and key neuropeptides (Alsop and Vijayan, 2009). Corticotropin-releasing hormone (CRH) from the nucleus preopticus (NPO), homologous to the mammalian paraventricular nucleus (PVN) (Herget et al., 2014), triggers the release of adrenocorticotropic hormone (ACTH) (Alderman and Bernier, 2009). ACTH then stimulates the interrenal gland, the fish analogue of the adrenal gland, to release GCs like cortisol, a process known as glucocorticoid reactivity (GC_R). GCs target the brain and periphery, facilitating stress adaptation (Munck et al., 1984; Chrousos, 1998).

Key to GC regulation is the negative feedback loop (GC-NF), where GCs inhibit the release of CRH and ACTH (Dallman and Yates, 1969). This mechanism is crucial for normalizing GC levels post-stress (de Kloet et al., 1998; Dallman et al., 1994; Charmandari et al., 2005) and maintaining neuroendocrine balance (Lightman and Conway-Campbell, 2010; Gjerstad et al., 2018). It prevents harm from excessive GCs (McEwen, 2007; Ulrich-Lai and Herman, 2009; Lupien et al., 2009; de Kloet and Joëls, 2023). GC-NF operates across different timescales (Kim and Iremonger, 2019; Shipston, 2022), involves both genomic and non-genomic pathways (Dallman, 2005; Evanson et al., 2010; Tasker and Herman, 2011; Joëls et al., 2013), and relies on GCs binding to mineralocorticoid (MR) and glucocorticoid (GR) receptors in the hypothalamus and pituitary to inhibit CRH and ACTH release. In

zebrafish larvae, GR and MR genes are expressed at 2 and 4 days post-fertilization (dpf), respectively (Alsop and Vijayan, 2008). This early expression, along with evidence that disruptions in GR function and changes in endogenous cortisol levels affect baseline cortisol and GC_R (Griffiths et al., 2012; Wilson et al., 2013; Castillo-Ramírez et al., 2019; Nagpal et al., 2024; De Marco et al., 2013), is crucial for comparing cortisol response dynamics between zebrafish larvae and conventional mammalian models. However, certain key aspects remain unclear. Specifically, the refractory period in cortisol response to repeated stress—i.e., the interval between stressor exposures that allows for cortisol elevation—has not been fully defined. Additionally, the effects of GR blockage on GC_R dynamics need further investigation to clarify the role of GR in the early phase of GC-NF. We addressed these issues by using controlled water vortices as stressors (Castillo-Ramírez et al., 2019), combined with GR blockage and measurements of GC_R through single and repeated stress protocols.

Results

Cortisol reactivity in response to single and repeated stress

Zebrafish larvae adapt to a water vortex by orienting against the current (Olszewski et al., 2012), making it a natural stressor (Castillo-Ramírez et al., 2019). This adaptation reflects their ability to align swimming behaviour with flow strength, imposing high energy demands and activating the HPI axis. Using vortex-induced forced swimming, we assessed baseline and post-stress whole-body cortisol in 6 dpf larvae. Exposed to 3-minute vortex flows of low or high strength (see Methods), whole-body cortisol was measured at 10, 20, 30, and 40 minutes post-exposure (Fig. 1A). Confirming prior findings (Castillo-Ramírez et al., 2019), we found a positive correlation between vortex strength and GC_R (Fig. 1B). Whole-body cortisol levels were elevated 10 minutes post-exposure and gradually decreased thereafter. For

both low and high vortex strengths, cortisol levels returned to baseline within 30 and 40 minutes, respectively (Fig. 1C). Therefore, higher cortisol responses do not hinder a rapid return to baseline whole-body cortisol after acute stress. Cortisol decreases steadily after exposure, irrespective of peak stressor-derived levels. We then explored the link between GC_R and a single homotypic stress event, varying the time interval between first and second vortices at 30, 60, or 120 minutes (Fig. 1D). Larvae showed no significant cortisol increase when exposed to a second vortex, regardless of its strength, 30 minutes after the initial exposure. However, at both 60 and 120 minutes post-initial exposure, they showed a GC_R level correlating with the strength of the second vortex (Fig. 1E, F).

Cortisol response to 30-min homotypic stress in Mifepristone-treated larvae

We then measured whole-body cortisol levels in separate groups of unstressed and stressed 6 dpf larvae treated with either DMSO or a combination of DMSO and Mifepristone, a verified zebrafish GR antagonist (Weger et al., 2012). Stressed larvae experienced either a single 3-minute high-strength vortex, with cortisol levels measured at 10, 30, and 40 minutes post-initial exposure (Fig. 2A, top), or two vortices spaced 30 minutes apart (Fig. 2A, bottom). Mif-treated larvae showed lower baseline whole-body cortisol levels than controls (Fig. 2B). Additionally, while DMSO-treated larvae showed a gradual post-exposure cortisol decrease, reaching baseline levels at 40 minutes, Mif-treated larvae retained cortisol levels above baseline at the same time point (Fig. 1B, C). Following exposure to a second high-strength vortex 30 minutes post-initial exposure, Mif-treated larvae showed a significant cortisol increase compared to the cortisol levels at the same post-initial exposure time (40’), unlike DMSO-treated or untreated larvae, which did not show a significant GC_R to the second vortex (Fig. 2D).

Discussion

We further examined GC_R dynamics and cortisol feedback in stressed zebrafish larvae, focusing on the refractory period in GC_R to repeated stress and the role of GR in the early phase of GC-NF. We observed elevated cortisol levels 10 minutes after the onset of stress, with levels returning to baseline 30-40 minutes later, depending on the strength of the stressor. Our measurements of cortisol at 5, 10, and 15 minutes following vortex exposure also revealed a peak at 10 minutes post-vortex onset (data not shown). This rapid increase in cortisol is consistent with previous findings, which report elevated whole-body cortisol levels around 10 minutes after stress in zebrafish larvae exposed to brief vortices, mild electric shocks, and air exposure (Faught and Vijayan, 2022; Hare et al., 2021; Steenbergen et al., 2012). In response to a repeated stressor, cortisol levels rose above baseline only when the second stressor occurred 60 or 120 minutes after the first, but not with a 30-minute interval. These results indicate a rapid cortisol-mediated feedback loop with a refractory period of at least 30 minutes. The return to baseline cortisol levels was delayed, and the refractory period was suppressed when larvae were treated with the GR antagonist Mifepristone, indicating GR-dependent feedback. These findings are crucial for validating the relevance of zebrafish as a stress research model.

Previous evidence links GR to GC-NF in zebrafish larvae. Early cortisol balance relies on both GR and MR (Alsop and Vijayan, 2009; Faught and Vijayan, 2018). GR-deficient larvae show no cortisol response to stress, with steroid levels remaining high compared to controls. Cortisol fails to return to baseline in these larvae, indicating impaired negative feedback. These mutants also show increased HPI axis activity, reduced cortisol suppression by dexamethasone, and elevated CRH and ACTH levels (Griffiths et al., 2013; Ziv et al., 2013; Fonseka et al., 2016). Acute stressors, such as light pulses, initially raise cortisol levels, followed by a decrease, consistent with responses to other stressors (De Marco et al., 2013;

De Marco et al., 2014; De Marco et al., 2016). However, repeated light pulses at 30-minute intervals do not increase cortisol levels after the initial pulse, whereas pre-incubation with Mifepristone elevates cortisol for both the initial and subsequent pulses (De Marco et al., 2013). Unlike knockout studies, which are limited by the absence of GR, using a GR blocker enables direct measurement of GR inhibition's effects on GC_R dynamics.

A functional GC-NF in zebrafish larvae predicts two specific outcomes. Firstly, encountering the same acute stressor twice will diminish GC_R, with the reduction depending on the interval between exposures: shorter intervals yield greater reductions. We found that a second exposure to the same stressor diminished GC_R, with shorter intervals yielding greater reduction. Secondly, during the refractory period, larvae treated with a GR blocker will show a higher GC_R to the second exposure compared to controls. Larvae treated with Mifepristone showed prolonged cortisol release but retained GC_R, unlike controls. Confirming both predictions is important, as reduced GC_R to closely spaced stressors may involve neural mechanisms independent of GCs, similar to findings in mice. In mice, stress familiarity diminishes CRH neuron responses independently of GC-NF, potentially reducing HPA axis activation through CRH neural response adaptation (Kim et al., 2019). This possibility needs further investigation in zebrafish. Nevertheless, confirming these two outcomes suggests that early-phase GR-mediated feedback reduces larval GC_R during subsequent exposure to homotypic stress 30 minutes later.

While Mifepristone is a known zebrafish GR antagonist (Weger et al., 2012; De Marco et al., 2013), using other antagonists and measuring GR-responsive genes could provide more details about GC_R dynamics with blocked GR signalling. Mif-treated larvae showed lower baseline whole-body cortisol than controls, contrary to expectations that blocked GR feedback would elevate basal levels (Faught and Vijayan, 2018; Ziv et al., 2013; Fig. 2). More experiments are needed to explore how GR antagonists affect baseline cortisol based on age, dosage, and incubation time. In zebrafish larvae, MR signalling also regulates

the HPI axis. MR mutants do not show differences in baseline or maximum post-stress cortisol levels, but they show a delayed cortisol rise and prolonged elevation beyond 30 minutes post-stress compared to controls (Faught and Vijayan, 2018). Further research is needed to clarify the separate and combined roles of GR and MR in early GC-NF. Additionally, studying the age-dependent and long-term effects of GR blockage on stress physiology and replicating these experiments with heterotypic stress using pH drop and/or hyperosmotic medium (Herget et al., 2023) would be valuable.

Data on the relationship between GR, GC-NF, and GC_R during repeated stress in rodents is limited. Past rodent studies examined ACTH and corticosterone secretion after restraint stress (De Souza and Van Loon, 1982). After a single stress event, ACTH peaked quickly and normalized within 30 minutes, while corticosterone peaked at 15-30 minutes and returned to baseline within 60-90 minutes. These responses were consistent after repeated stress every 90 minutes, but closer stress events reduced the corticosterone response, indicating a narrower adrenocortical window. Research indicates that GR activation regulates stress-induced HPA axis activity (Ratka et al., 1989; Sapolsky et al., 2000). Local GR knockdown in the PVN increased stress-induced ACTH and corticosterone (Solomon, 2015), and genetic manipulations of GR elevated stress-induced GCs and disrupted GC-NF (Laryea et al., 2013). Thus, PVN GR mediates early-phase feedback, controlling the duration and intensity of GC secretion post-stress. GR activation across the brain supports arousal, cognitive performance, and modulates immune and inflammatory responses in response to metabolic demands (de Kloet, 2023), highlighting the intricate nature of GC-NF during acute stress.

Our findings are consistent with GC_R dynamics seen in conventional mammalian models, showing similar cortisol peak times in response to stress. They validate the high-throughput forced swim procedure for repeated stress and enhance our understanding of cortisol regulation in zebrafish larvae, which are well-suited for assessing stress response

mechanisms in line with the 3Rs principle (e.g., Eachus et al., 2021; Swaminathan et al., 2023; De Marco et al., 2016; vom Berg-Maurer et al., 2016; de Abreu et al., 2021). Together, the results provide a more detailed framework for studying GC regulation in the hypothalamus and anterior pituitary post-acute stress using non-invasive live imaging. We anticipate that this framework will be valuable for exploring the overlap between early life stress and HPI axis programming.

Methods

Zebrafish husbandry and handling

Zebrafish breeding and maintenance were performed under standard conditions (Westerfield, 2000). 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 dpf, the E2 medium was renewed, and chorions and debris were removed from the dishes. Experiments were carried out with 6 dpf larvae. Zebrafish experimental procedures were performed according to the guidelines of the German animal welfare law and approved by the local government (Regierungspräsidium Karlsruhe; G-29/12).

Water vortex flows

We used water vortex flows in a high-throughput fashion to induce rheotaxis and cortisol increase. Groups of thirty larvae in 35 mm Petri dishes with 5 ml of E2 medium were exposed to vortex flows caused by the spinning movements of small magnetic stir bars (6 x 3mm, Fischerbrand, #11888882, Fisher scientific, Leicestershire, UK) inside the dishes. The Petri dishes, each with a single stir bar, were positioned on magnetic stirrer plates (Variomag, Poly 15; Thermo Fisher Scientific, Leicestershire, UK) and kept at 28°C inside an incubator

(RuMed 3101, Rubarth Apparate GmbH, Laatzen, Germany). Larvae were exposed to vortex flows induced by the magnetic field inversions of the stirrer plate for 3-minute periods, at 130 (low strength) or 330 (high strength) revolutions per minute (rpm). The magnetic field inversions of the stirrer plate, *per se*, do not alter the level of whole-body cortisol in larval zebrafish, and we used a maximum of 330 rpm to avoid potential ceiling effects caused by the maximum levels of vortex-dependent cortisol increase (Castillo-Ramírez et al., 2019). Larvae were exposed to the 3-min vortex either once or twice, with the second exposure occurring 30, 60, or 120 minutes after the first exposure, depending on the experimental series. They were subsequently used for cortisol detection (see below). Basal levels of cortisol were measured before first-time exposure to vortex flows.

Whole-body cortisol

The procedures and the home-made ELISA used for cortisol measurements were as described previously (Yeh et al., 2013). After exposure to the vortex, groups of thirty larvae were immobilized in ice water. Unexposed larvae were collected after equal handling, omitting stressor exposure. The samples were then frozen in an ethanol/dry-ice bath and stored at −20 °C for subsequent extraction. Each replicate consisted of a well with 30 larvae. Cortisol extraction and detection were carried between 10:30 and 11:30 hours.

Mifepristone incubation

Before exposure to vortex, larvae were incubated for 2 hours in 1 µM Mifepristone (RU486, Sigma-Aldrich) dissolved in E2-Medium with 0.1% DMSO. This concentration has been shown to abolish a genomic GC response signal (Weger et al., 2012).

Independent sampling

Cortisol measurements were conducted on distinct groups of larvae, all subjected to the same treatment, ensuring fully independent samples. In every instance, measurements were taken from larvae housed in wells containing a total of thirty individuals. Consequently, the larval density per well remained a constant factor during vortex flow exposure for cortisol measurements, preventing any confounding effects. For each cortisol measurement, all thirty larvae within a well were utilized. Each replication was entirely independent from the others, thus avoiding pseudo-replication.

General experimental design and statistical analyses

In all experiments, treatments were randomly assigned to replicates, and blinding was implemented. For cortisol measurements, the initial experimenter conducted the treatments, collected, and labelled the samples. Subsequently, a second experimenter performed measurements on the labelled samples, assigning them new labels. The first experimenter then quantified cortisol using the newly encoded samples. All single measurement points, along with means or medians based on the data distribution, are presented in both Fig. 1 and Fig. 2. No data points or samples were excluded from analysis. Statistical methods to predetermine sample size were not necessary. Our sample sizes are similar to (or larger than) those generally employed in the field, and based on previous publications (Castillo-Ramírez et al., 2019; De Marco et al., 2016; Yeh et al., 2013). Data were tested for normality and homoscedasticity using the Shapiro-Wilk and KS normality tests and the Brown–Forsythe and Bartlett's tests, respectively. For two-group comparisons, we used Mann-Whitney tests (for pairwise comparisons with at least one non-normally distributed dataset) or unpaired Student's *t*-tests (two-tailed) (for normally distributed datasets). For multiple group comparisons in Fig. 1E, we used Kruskal-Wallis tests followed by Dunn's multiple comparison tests against initial exposure. We also used one sample Wilcoxon signed rank tests or one sample *t*-tests against a median or mean fold change of 1, respectively (Fig. 1C, F,

and Fig. 2C, D). Statistical analyses were performed using MS-Excel (Microsoft Corp; Redmond, WA, USA) and Prism 10.2.0 (Graphpad Software Inc, San Diego, CA, USA).

Data availability

The experimental datasets presented in this study are available in online repositories, accessible upon request from the authors.

Ethics

The present study was reviewed and approved by Regierungspräsidium Karlsruhe; G-29/12.

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Author Contributions

Luis A. Castillo-Ramírez, Conceptualization, Data curation, Formal analysis, Investigation, Validation, Methodology, Writing - original draft; Soojin Ryu, Conceptualization, Resources, Supervision, Funding acquisition, Methodology, Project administration, Writing – review; Rodrigo J. De Marco, Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Validation, Methodology, Data curation, Formal analysis, Visualization, Project administration, Writing - original draft, Writing - review and editing.

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Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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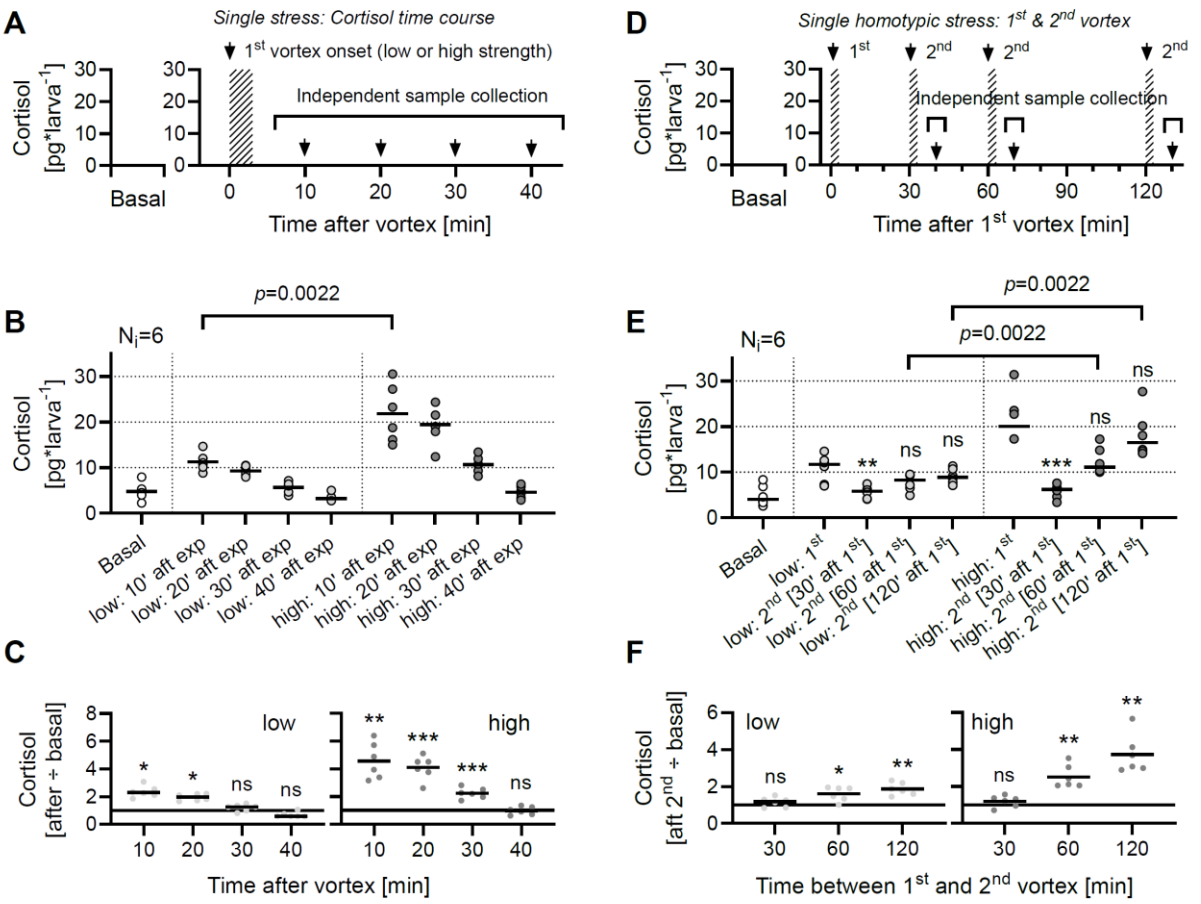
Figure legends

Figure 1. Cortisol reactivity under single and homotypic stress. (**A**) Protocol for assessing basal and post-vortex cortisol levels, measured at 10, 20, 30, and 40 minutes after exposure. (**B**) Basal and post-vortex cortisol levels. $p = 0.0022$ (U = 0) by Mann-Whitney test. White, light grey, and dark grey represent basal cortisol and data from low and high vortex strength, respectively, for all plots. N per group = 6. (**C**) Cortisol levels post-exposure relative to basal cortisol (data from B). Left, 10': $*p = 0.03$ (W = 21), 20': $*p = 0.03$ (W = 21), 30': $p = 0.16$ (W = 15), 40': $p = 0.06$ (W = -19). Right, 10': $**p = 0.001$ ($t_{(5)} = 6.7$), 20': $***p = 0.0003$ ($t_{(5)} = 8.7$), 30': $***p = 0.0006$ ($t_{(5)} = 7.7$), 40': $p = 0.81$ ($t_{(5)} = 0.25$). (**D**) Protocol for measuring basal cortisol and GC_R in response to homotypic stress at different intervals between two vortices (30, 60, or 120 minutes). (**E**) Basal cortisol and GC_R in response to a second vortex at varying intervals. 60': $p = 0.0022$ (U = 0), 120': $p = 0.0022$ (U = 0) by Mann-Whitney tests. For low and high vortex strength, $p = 0.01$ (H = 11.3) and $p = 0.0004$ (H = 18.4) by Kruskal-Wallis tests, respectively. Followed by Dunn's multiple comparison tests against initial exposure, with adjusted *p* values; low, 30': $**p = 0.004$, 60': $p = 0.39$, 120': $p > 0.99$; high, 30': $***p = 0.0002$, 60': $p = 0.07$, 120': $p > 0.99$. (**F**) Cortisol levels post-second exposure relative to basal cortisol (data from E). Left, 30': $p = 0.22$ ($t_{(5)} = 1.4$), 60': $*p = 0.01$ ($t_{(5)} = 3.8$), 120': $**p = 0.002$ ($t_{(5)} = 6.3$). Right, 30': $p = 0.21$ ($t_{(5)} = 1.4$), 60': $**p = 0.002$ ($t_{(5)} = 6.1$), 120': $**p = 0.001$ ($t_{(5)} = 6.4$). (**C**, **F**) *p* values by one sample Wilcoxon signed rank tests or one sample *t*-tests against a median or mean fold change of 1, respectively.

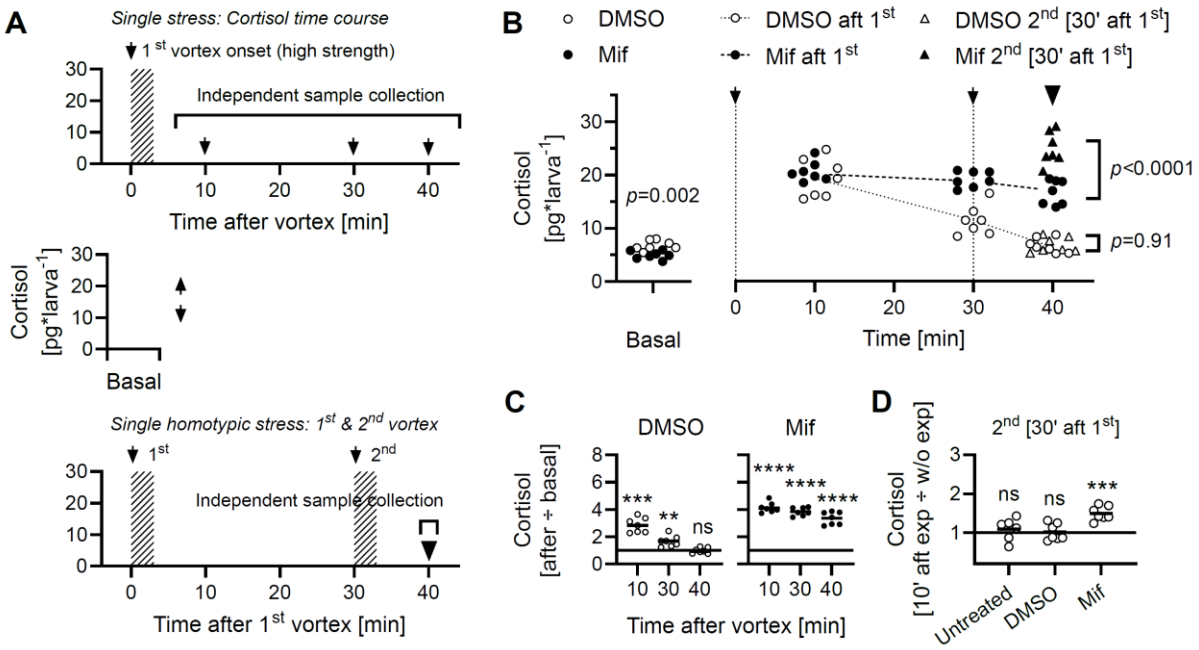
Figure 2. Cortisol response to 30-min homotypic stress in Mifepristone-treated larvae. (**A**) Protocol for assessing basal cortisol, post-vortex cortisol levels (measured at 10, 30, and 40 minutes post-exposure), and GC_R in response to a second high-strength vortex (with a 30-minute interval) in DMSO or DMSO + Mifepristone (Mif) incubated larvae. For all plots,

except D, white represents DMSO group data, and black represents Mif group data. N per group = 7, except for the untreated group (D), where N = 6. (**B**) Baseline cortisol, post-first vortex levels, and cortisol in DMSO- and Mif-treated larvae after a second vortex at 30 minutes post-initial exposure, compared to independently collected cortisol levels at the same time (40'). $p = 0.002$ ($t_{(12)} = 4.01$), $p < 0.0001$ ($t_{(12)} = 5.74$) $p = 0.91$ ($t_{(12)} = 0.12$) by unpaired two-tailed *t*-tests. (**C**) Cortisol levels post-first vortex relative to baseline cortisol (data from B). Left, 10': ***$p = 0.0001$ ($t_{(6)} = 9.0$), 30': **$p = 0.005$ ($t_{(6)} = 4.4$), 40': $p = 0.84$ ($t_{(6)} = 0.20$). Right, 10': ****$p < 0.0001$ ($t_{(6)} = 22.1$), 30': ****$p < 0.0001$ ($t_{(6)} = 25.1$), 40': ****$p < 0.0001$ ($t_{(6)} = 13.3$). (**D**) Cortisol level 10 minutes after the second vortex compared to the level at the same time without the vortex (data from B, 40'). Untreated: $p = 0.49$ ($t_{(5)} = 0.74$), (N = 6), DMSO: $p = 0.86$ ($t_{(6)} = 0.17$), 40': ***$p = 0.0001$ ($t_{(6)} = 7.24$). (**C**, **D**) *p* values by one sample *t*-tests against a mean fold change of 1.

Figure 1


A
Single stress: Cortisol time course
1st vortex onset (low or high strength)
Independent sample collection
Cortisol [pg*larva-1]
Basal
Time after vortex [min]
D
Single homotypic stress: 1st & 2nd vortex
1st
2nd
2nd
2nd
Independent sample collection
Cortisol [pg*larva-1]
Basal
Time after 1st vortex [min]
B
p=0.0022
Ni=6
Cortisol [pg*larva-1]
Basal
low: 10' aft exp
low: 20' aft exp
low: 30' aft exp
low: 40' aft exp
high: 10' aft exp
high: 20' aft exp
high: 30' aft exp
high: 40' aft exp
E
p=0.0022
p=0.0022
Ni=6
Cortisol [pg*larva-1]
Basal
low: 1st
low: 2nd [30' aft 1st]
low: 2nd [60' aft 1st]
low: 2nd [120' aft 1st]
high: 1st
high: 2nd [30' aft 1st]
high: 2nd [60' aft 1st]
high: 2nd [120' aft 1st]
C
Cortisol [after ÷ basal]
low
high
Time after vortex [min]
F
Cortisol [aft 2nd ÷ basal]
low
high
Time between 1st and 2nd vortex [min]

511 **Figure 2**


A
Single stress: Cortisol time course
1 st vortex onset (high strength)
Independent sample collection
Time after vortex [min]
Cortisol [pg*larva-1]
Basal
Single homotypic stress: 1st & 2nd vortex
1st
2nd
Independent sample collection
Time after 1st vortex [min]
B
DMSO
Mif
DMSO aft 1st
Mif aft 1st
DMSO 2nd [30' aft 1st]
Mif 2nd [30' aft 1st]
Cortisol [pg*larva-1]
p=0.002
p<0.0001
p=0.91
Basal
Time [min]
C
DMSO
Mif
Cortisol [after ÷ basal]
***
**
ns
****
****
****
Time after vortex [min]
D
2nd [30' aft 1st]
Cortisol [10' aft exp ÷ w/o exp]
ns
ns
***
Untreated
DMSO
Mif