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Review

The Potential of Zebrafish Larvae and Water Vortex Protocols in Stress Biology

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Abstract: Stress responses enable vertebrates to adapt to environmental challenges while maintaining homeostasis. Zebrafish larvae are a valuable model for studying stress regulation due to their genetic accessibility and rapid development. This review examines the integration of zebrafish larvae with water vortex protocols to investigate hypothalamic–pituitary–interrenal (HPI) axis functionality during early development, advancing stress research while adhering to the 3Rs principle. Key publications are reviewed to discuss the potential of water vortices in zebrafish larvae for studying stress responses. These purely physical stressors exploit the innate positive rheotropism of developing zebrafish, offering precise control over timing and strength while avoiding confounding factors associated with chemical or biological interventions. The approach enables reproducible assessments of stress responses. The reviewed publications show advances in understanding cortisol response dynamics, glucocorticoid feedback, and early-life stress-induced changes in HPI axis function. Key findings include detailed cortisol patterns after acute stress, rapid glucocorticoid receptor-mediated feedback regulating cortisol levels, developmental shifts in HPI axis sensitivity, and reduced cortisol reactivity following early-life challenge (ELC). Vortex-driven ELC affects cortisol regulation, neuropeptide expression in the nucleus preopticus, and stress-related gene transcription. Combining zebrafish larvae and vortex protocols provides a robust and innovative platform for investigating stress biology. This approach leverages active, demanding behaviour to study stress mechanisms under controlled conditions, yielding insights with broad applications across vertebrate models while supporting the 3Rs principle. Future studies can build on these findings to address unresolved questions in stress regulation and enhance our understanding of adaptive physiological mechanisms.

Keywords: zebrafish larvae; HPI axis; water vortex protocols; cortisol dynamics; glucocorticoid feedback; early-life challenge (ELC); stress regulation; high-throughput stress models; 3Rs principle

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1. Introduction

Stress responses are essential biological mechanisms that enable vertebrates to adapt to environmental challenge while preserving a physiological and behavioural balance [1]. These mechanisms are coordinated by the hypothalamic–pituitary–adrenal (HPA) axis in mammals [2,3] and its homologue, the hypothalamic–pituitary–interrenal (HPI) axis, in fish. At the core of the HPA/I axis are glucocorticoids (GCs), essential hormones that regulate stress-related processes to support adaptation to adverse conditions [4–8].

In recent years, zebrafish larvae (*Danio rerio*) have emerged as a powerful model for studying stress biology, owing to their unique combination of genetic accessibility, rapid

development, and transparent body. These features facilitate precise investigations into the early-life dynamics of HPI axis regulation [9–11]. Additionally, the introduction of high-throughput water vortex protocols has expanded the experimental toolkit for stress research, offering a purely physical, controllable stressor that avoids potential confounds associated with chemical or biological interventions. By exploiting the innate behaviour of rheotaxis, this approach achieves a remarkable level of consistency and reproducibility while adhering to the 3Rs principle: Replacement, Reduction, and Refinement. Replacement is achieved by using subjects at developmental stages prior to independent feeding—for instance, employing 5 days post-fertilisation (dpf) larvae that are not classified as protected—while some experimental designs may also incorporate older larvae as needed. Reduction is achieved through the high reproducibility of rheotaxis-based vortex flow assays, enabling low coefficients of variation across experimental groups with fewer iterations. Refinement is ensured by the non-invasive nature of the method, which elicits a natural response without physical handling or direct stressors.

This review synthesises key findings from studies employing this methodology. It highlights advances in understanding cortisol response patterns, the role of glucocorticoid feedback mechanisms, and stress-induced transcriptional changes during early development. Beyond summarising these contributions, the review underscores the broader implications of integrating zebrafish larvae and water vortex protocols for experimental stress research, providing a framework with applicability across vertebrate models.

2. Early Life Adaptability of the Stress Response Machinery

A key feature of the HPA axis is its adaptability as an open system, continuously interacting with and responding to environmental inputs. This dynamic nature allows the organism to adjust to environments that disrupt homeostasis, facilitating protective adaptations [12,13]. During early development, when physiological and behavioural systems are still maturing, this flexibility is particularly crucial. The responsiveness of the HPA axis at this stage not only shapes immediate stress responses but is also thought to influence long-term stress-regulation capacity, potentially laying the foundation for resilience throughout life. Excessive or poorly timed stress increases allostatic load, accelerating maladaptive processes [14–17]. While the effects of stress in adulthood are often transient, early-life stress can significantly alter the development of bodily functions, leading to enduring behavioural changes [16,18–20]. These effects may emerge in childhood, adolescence, or adulthood, as the delayed impact of stress-induced brain alterations becomes evident [21–24]. Both pre- and postnatal environments shape stress regulation and long-term HPA axis function, although the mechanisms driving these effects remain unclear.

Investigating the early calibration of the stress response system is crucial for understanding how stress responsiveness develops and how early-life experiences influence future stress regulation. Zebrafish larvae provide unique advantages for this research. Their external development allows for detailed analysis of stress modulators during critical periods [25–29], enabling insights into GC regulation and stress-related pathologies [9,30,31]. By bridging gaps between zebrafish and mammalian stress models, studies using zebrafish larvae can advance both basic and translational research. Furthermore, their use aligns with the 3Rs principles, minimising reliance on higher vertebrates while supporting high-throughput, ethically sound experimentation.

3. Larval Zebrafish as a Model Organism for Stress Research

Larval zebrafish have emerged as a model for studying stress dynamics from early development, owing to their conserved stress response mechanisms, which closely resemble those in mammals. In mammals, stressors activate neurons in the paraventricular nucleus

(PVN) of the hypothalamus, releasing neurotransmitters such as corticotropin-releasing hormone (CRH), the primary activator of the HPA axis [13]. In zebrafish, the nucleus preopticus (NPO), homologous to the mammalian PVN [32], orchestrates similar stress-response processes. Upon stress onset, CRH binds to type 1 CRH receptors on pituitary cells, initiating cAMP and Ca^{2+} signalling pathways that precede hormone secretion [33]. This cascade stimulates the expression and release of adrenocorticotropic hormone (ACTH), derived from the precursor proopiomelanocortin (POMC), which signals the adrenal cortex in mammals and the interrenal tissue in zebrafish to produce and release GCs.

Zebrafish larvae possess a fully developed HPI axis, homologous to the mammalian HPA axis [25,26,32,34]. The interrenal organ, analogous to the adrenal gland in tetrapods, comprises aminergic chromaffin cells and steroidogenic interrenal cells, each originating from distinct embryological sources [35,36]. Cortisol, produced by steroidogenic interrenal cells, serves as the final effector of the HPI axis, playing a pivotal role in reestablishing homeostasis and mediating developmental transitions.

The early functional maturation of the zebrafish HPI axis makes it an ideal model for investigating the mechanistic aspects of vertebrate stress responses [37–42]. As a non-mammalian vertebrate model, zebrafish larvae offer several practical advantages. Zebrafish show high reproductive capacity, and their larvae are small, externally developing, and low-cost to maintain [43,44]. These characteristics make zebrafish larvae particularly well-suited for studying early HPI axis activity and adaptation in controlled environments, free from the confounding effects of prenatal stress or maternal care variations.

An additional advantage of zebrafish is their evolutionary history as teleost fish, which underwent a genome duplication event. This duplication resulted in two copies of some genes that are present as single copies in mammals [45]. While this can complicate gene function analyses, critical components of the HPI axis, such as CRH, ACTH, and glucocorticoid receptor (GR), exist as single functional genes in zebrafish [26]. In some cases, gene duplication can even be advantageous, particularly for studying genes whose null mutations are lethal in mammals.

Importantly, their externally developing embryos enable controlled exposure to physical and chemical environmental factors. Furthermore, the genetic accessibility of the zebrafish larva facilitates diverse assays to evaluate both physiological and behavioural stress responses.

4. Cortisol and Behavioural Responses to Stressors in Zebrafish Larvae

The secretion of GCs, such as cortisol, increases rapidly following the onset of stress, a process known as glucocorticoid reactivity (GC_R), and is vital for the ability of the organism to respond to challenge. GCs play a central role in facilitating the physiological adjustments required to cope with stress [5,6,12,46–48]. Zebrafish larvae, with their well-characterised cortisol response to environmental stressors, therefore serve as an excellent model for studying stress physiology during development. Over the past decade, we have systematically explored various stimuli that trigger cortisol release, including light pulses [34,49,50], hyperosmotic conditions [42,51,52], unpredictable and intense water movements [42,50,51], pH drops [42,50,52], and vortex-induced forced swimming [53–55].

Cortisol measurements in zebrafish larvae are not without challenge. Standard protocols typically require pooling multiple individuals—often 10–30 larvae per replicate (e.g., [25,50])—to obtain sufficient material for analysis, which limits the assessment of individual variability. Although this approach remains widely used, recent methods have enabled cortisol measurements from single larvae, thereby facilitating analyses of individual stress responses [56]. Moreover, while cortisol remains the primary stress marker

in larval zebrafish, other physiological indicators (e.g., [52,55]) remain comparatively underexplored.

Complementing our physiological studies, we have, over the past decade, systematically examined behavioural stress responses in zebrafish larvae. Our analyses have revealed reduced locomotion after sudden light exposure, avoidance of hyperosmotic areas, transient feeding suppression, increased swimming following mechanosensory disturbances, and altered movement patterns after pH drops. Additionally, we have documented how acute stress influences innate behaviours, including the optomotor reflex, positive mechanotaxis, and thermally guided space use, with moderate stress enhancing performance [34,42,49–52,57] (see below).

For example, larval zebrafish are highly sensitive to light stimuli. When briefly (~5 min) adapted to darkness, they show stable, discontinuous movement and respond predictably to brief light exposure with changes in locomotion. Initially, their movement sharply decreases at light onset, then increases after light offset, gradually returning to baseline levels. This rapid reduction in activity in response to external stimuli is typically considered a fear-related response, with many species releasing cortisol in reaction to fear-inducing situations. While developing optogenetic tools for stress research [34,49], we assessed how light exposure affects locomotion and cortisol levels in freely behaving larvae. Furthermore, since optogenetic photo-actuators respond to a range of light wavelengths, we tested whether zebrafish would show similar reactions to blue and yellow light pulses. We observed that both wavelengths elicited similar locomotion patterns. Notably, at 6 dpf, a square pulse of either blue or yellow light not only triggered locomotor reactions but also increased whole-body cortisol in a dose-dependent manner, depending on light power and exposure time. Cortisol levels peak 5–10 min after exposure and return to baseline within 30 min. This indicates that light changes can serve as a stress signal [34,49,50]. The response was not due to differences in wakefulness, as larvae exposed to constant white light or complete darkness displayed similar locomotor activity.

Exposure to a hyperosmotic medium, such as increased NaCl concentrations, can also act as a stressor in larval zebrafish, elevating whole-body cortisol in a concentration-dependent manner. Again, cortisol levels peak 5–10 min after exposure and return to baseline within 30 min [34,42,50–53]. Behaviourally, larvae actively avoid areas of sudden NaCl addition [51,52]. Notably, hyperosmotic exposure transiently suppress feeding, with higher NaCl concentrations (100 mM) delaying recovery compared to lower concentrations (50 mM). However, this stressor did not affect post-exposure locomotion, oxygen consumption rates, or visual responses, including reactions to light changes or optomotor stimuli [51]. While hyperosmotic stress triggers significant cortisol and behavioural changes, these findings indicate that locomotor activity, oxygen consumption, and visual processing remain unaffected.

Rapid water movements that disrupt normal swimming also elicit mechanosensory stress in larval zebrafish, increasing whole-body cortisol in a stimulus strength-dependent manner. This response can be triggered using a silica capillary attached to a computer-controlled piezo actuator, which generates lateral displacements (LDs) in the surrounding water. When exposed to these rapid water movements in an elongated swimming chamber, larvae rapidly increase their distance from the moving capillary and show increased locomotion proportional to the stimulus strength. The LD stimuli likely mimic natural predation cues, such as pressure waves from larger fish or approaching predators. Cortisol levels peak shortly after stimulation and return to baseline within 30 min, exposing the transient nature of this physiological response [34,42,50,51]. Similar to exposure to a hyperosmotic medium, low to moderate levels of mechanosensory stress can transiently

suppress feeding, with full recovery occurring 40–50 min later. Importantly, this type of stress does not affect post-exposure locomotion [51].

A sudden drop in the pH of the surrounding water poses a significant challenge to homeostasis in larval zebrafish, eliciting acute stress responses, including strength-dependent increases in whole-body cortisol levels [42,50,52]. Using custom-designed swimming chambers and computer-controlled perfusion systems, we investigated the effects of pH drops on larval behaviour and stress physiology. Avoidance tests showed that larvae rapidly altered their movement patterns and avoided areas where hydrochloric acid (HCl) solutions were introduced, in contrast to control conditions. Locomotion tests in a flowing-water setup revealed that larvae exposed to small pH drops, whether from low or high concentrations of HCl, showed distinct changes in movement dynamics during and immediately after exposure [52]. These behavioural and physiological responses highlight the sensitivity of larval zebrafish to pH fluctuations and their suitability for studying stress reactions triggered by environmental acidification.

5. Stress Levels and Performance in Innate Behaviour

The four acute stress protocols outlined above—sudden light pulses, hyperosmotic conditions, fast water movements, and pH drops—have, for the first time, revealed the relationship between stress levels and performance in innate behaviours. Zebrafish larvae show a robust optomotor reflex in response to large-field visual displacements, rely on hydrodynamic sensing for mechanotaxis, and can select optimal conditions within a thermal-gradient environment [50,51,57]. By integrating these acute stress protocols with novel behavioural assays, we analysed the performance of stressed larvae in behaviours driven by visual, hydrodynamic, and thermal cues.

In the first assay, we tested the optomotor reflex by exposing larvae to visual field displacements created by ventrally displayed moving dots. Larvae swim freely and individually in a rectangular chamber and show no preferred direction when the dots are stationary but swim in the same direction when the dots move in parallel. When the larvae reach the chamber's far end, the dots shift to the opposite end, prompting the larvae to align and swim with them [51]. 'Latency' (the time between the start of alignment and reaching the opposite end) serves as a measure of response strength, with lower latency indicating stronger responses. In the second assay, we tested hydrodynamic responses using controlled minute water motions (mWMs) at 5 Hz. Larvae respond to these non-stressful mWMs with a pronounced reduction in locomotion, accompanied by positive taxis toward the stimulus source [42,57,58]. We quantified this behaviour by measuring the distance swum before and during exposure, using 'fold change in motion' as a response strength indicator—larger reductions in movement correspond to stronger responses. In the third assay, we studied the ability of freely swimming larvae to avoid water temperatures above their preferred range by monitoring the time spent in each quadrant of a chamber with a stable temperature gradient. We quantified 'differential space use' (the difference in time spent in the low- and high-temperature quadrants) as an estimate of thermal sensitivity and stressor avoidance [50].

To evaluate the effects of acute stress on these behaviours, we compared performance across larvae with baseline, moderate, and high cortisol levels induced by the four previously described stressors. These stressors—light pulses, hyperosmotic conditions, intense water movements, and pH drops—elicit intensity-dependent cortisol responses, peaking 10 min after exposure. We achieved moderate cortisol levels by selecting stimulus intensities that produced responses intermediate between the lowest and highest extremes [50]. The results revealed a non-linear relationship between stress level and performance: moderate stress enhanced performance, while higher stress did not. Under moderate stress,

performance improved by factors of 1.5, 1.8, and 2 in visual, hydrodynamic, and thermal responses, respectively [50]. These findings are remarkable in identifying a non-linear stress-performance relationship at an early developmental stage, with an inverted-U curve linking acute stress to performance, a pattern likely common among vertebrates. This relationship may have evolved to cope with environmental variability and competition, with moderate stress enhancing survival during early development by improving responses to predation risks. Understanding how this relationship changes with maturation is essential, as behaviour and fitness are shaped by genetics, ontogeny, and developmental environments. While stress responses are often considered beneficial and may be linked to epigenetic programming, direct evidence connecting early HPA/I activation patterns to performance, resilience, and fitness is still lacking.

In line with these findings, evidence indicates that early-life stress can shape HPA axis function, with early adversity altering GC regulation and coping capacity later in life [59,60]. However, the mechanisms by which active responses to early stress recalibrate HPA axis functionality remain poorly understood. Investigating these mechanisms requires robust stress protocols capable of eliciting controlled and reproducible GC_R during early developmental stages. Such protocols are essential for advancing our understanding of how early stress experiences influence long-term resilience and fitness.

6. Controlled Water Vortices for Stress Protocols in Zebrafish Larvae

Larval zebrafish are uniquely suited for developing these robust stress protocols and for studying early stress experiences and their impact on HPA/I axis function. Their transparency enables non-invasive imaging and optogenetics, while their small size facilitates high-throughput screening. Combined with an extensive array of genetic and epigenetic tools, including proteomics, zebrafish larvae represent an exceptional model for exploring the physiological and molecular mechanisms underlying early-life stress.

As mentioned earlier, we identified light pulses, hyperosmotic conditions, intense water movements, and pH drops as effective stressors capable of eliciting robust, stressor strength-dependent cortisol responses. These hormonal responses are accompanied by measurable behavioural changes, including reversible shifts in feeding drive and increased responsiveness under moderate stress. These stressors offer precise control over onset, offset, and intensity during short-term exposures, aligning well with the zebrafish's suitability for high-throughput and finely tuned experimental designs. However, their use in long-term protocols poses challenges. Prolonged squared light pulses and dark adaptation may interfere with natural light/dark cycles, hyperosmotic conditions and acidification can affect survival rates, and intense, unpredictable water movements complicate standardisation.

A promising alternative to traditional stressors involves controlled vortex flows, which mimic natural environmental challenges while offering precise experimental control. In response to vortex flows, zebrafish larvae display rheotaxis, an innate behaviour where they orient against the current to maintain their position [61]. This behaviour imposes significant energy demands and activates the HPI axis. Although the metabolic and cardiorespiratory consequences of vortex exposure remain underexplored, the observed cortisol elevation indicates substantial energy expenditure and physiological stress [53–55].

Notably, several studies have applied mechanosensory stress in larval zebrafish using swirling protocols in which larvae are shaken within confined tubes rather than engaging in forced swimming via rheotaxis [27,62,63]. For instance, a 30 s swirling period at 600 revolutions per minute (rpm) in a glass beaker effectively activated cortisol-catabolic pathways in 5 dpf larvae, while a 1 min swirling period at 250 rpm in Falcon tubes elicited cortisol responses and elucidated receptor-specific contributions to stress regulation [27,62]. Similarly,

a 1 min vortex at 200 rpm in centrifugal tubes did not significantly alter cortisol, glucose, or lactate levels, suggesting that lower vortex speeds may activate alternative, cortisol-independent mechanisms—potentially involving catecholaminergic signalling—that drive behavioural and metabolic adaptations [63]. Collectively, these protocols have shown that strong water motions can effectively induce mechanosensory stress and have contributed significant findings to the understanding of stress physiology in larval zebrafish [27,62,63]. In this context, controlled water vortex protocols that evoke rheotaxis—a natural, energy-intensive counter-swimming behaviour—represent a complementary approach, offering a defined and reproducible environmental challenge for further exploring early-life stress physiology.

In laboratory settings, zebrafish larvae are typically maintained in highly controlled environments, with minimal environmental variability. Factors such as temperature, illumination, and food availability are precisely regulated, providing stable conditions that reduce extraneous influences on behaviour and physiology. This precise control makes zebrafish larvae exceptionally well-suited for studying the onset and dynamics of HPI axis calibration under defined environmental challenges. Within this framework, directional water currents offer a reproducible method to introduce physical challenges, prompting larvae to adjust their swimming to match flow strength. Vortex flows calibrated at specific rpm reliably induce rapid and pronounced cortisol increases, making them a valuable tool for stress research [53–55].

Vortices offer distinct advantages as stressors in controlled environments. Unlike those that alter physicochemical factors such as salt concentration or pH, vortex flows elicit stress responses through innate, reproducible behaviours, reducing variability in experimental outcomes. Furthermore, cortisol levels rise proportionally with vortex strength (rpm), reflecting the increased swimming effort required to counteract the flow [53]. This predictable relationship between vortex intensity and GC_R enables precise categorization of stress levels, establishing vortex flows as an effective tool for studying both prolonged exposure and repeated stress paradigms.

7. High-Throughput Forced Swimming Induction

As a first step in developing a high-throughput forced swimming protocol using water vortices, we examined the relationship between vortex strength, larval movement, and stress responses. Groups of larvae in Petri dishes containing a small volume of medium were exposed to water vortices of varying strength and duration, generated by small, coated magnetic stir bars placed inside the dishes. The controlled spinning of these stir bars, measured in rpm, created water currents that stimulated rheotaxis, requiring the larvae to swim against the flow, activating their HPI axis and eliciting cortisol increases. To quantify vortex strength, we tracked the paths (x–y coordinates) of anaesthetised larvae (unable to swim) exposed to increasing rpm. These observations confirmed that higher rpm caused anaesthetised larvae to move at greater speeds and cover larger distances from the vortex source. Based on these measurements, vortices were categorised as low, medium, or high strength. Freely swimming larvae responded to these vortices with positive rheotaxis, orienting against the current and adjusting their swim bouts and turns to compensate for vortex strength. They avoided the strongest currents by positioning themselves farther from the vortex source. These behaviours were energetically demanding, as evidenced by a proportional increase in whole-body cortisol with increasing vortex strength [53].

This protocol is highly versatile: short exposures (1–3 min) induce peak stressor-mediated cortisol responses, while prolonged exposures (up to 9 h at moderate vortex strength) provide extended stressor presentations compatible with a typical light/dark cycle, during which larvae consistently engage in positive rheotaxis. After exposure, larvae

can be immobilised in ice water for subsequent cortisol or behavioural measurements. Importantly, this protocol eliminates confounding variables associated with magnetic field inversions. Basal cortisol levels in larvae exposed to magnetic field inversions without vortex flows remain unchanged, confirming that the stress response is specifically induced by vortex flows rather than the magnetic fields themselves [53].

Building on this versatility, high-throughput forced swimming offers precise and adaptable methods for implementing repeated and long-term stress protocols in zebrafish larvae. For short-term exposures, vortex strength can be fine-tuned by adjusting duration and rpm and validated through cortisol measurements taken 10 min post-exposure. For example, a 3 min exposure at a calibrated rpm reliably activates the HPI axis without driving whole-body cortisol to its maximum levels 10 min post-exposure. Instead, cortisol increases to intermediate levels, within the range observed across different exposure durations (1, 2, or 3 min) and rpm settings. This controlled modulation is essential for repeated vortex applications, as the cumulative effects of repeated stress exposures depend on the magnitude of each individual response, which is shaped by dynamic regulatory mechanisms such as feedback inhibition. The ability to remotely control vortex initiation and cessation ensures minimal disturbance to the controlled environment of the larvae within incubators. For long-term protocols, moderate vortices can be sustained over extended periods, aligning with a typical light/dark cycle. During a 9 h exposure, for example, whole-body cortisol levels rise sharply following vortex initiation, remain elevated for approximately 4 h, and return to baseline by the 6 h mark. Larvae consistently show positive rheotaxis throughout the exposure, maintaining orientation against the current even during the final hours [53].

8. Cortisol Response to Vortex Stress: Early Responsiveness and Feedback Regulation

Using high-throughput vortex protocols, we examined developmental changes in GC_R during early larval stages of zebrafish [53]. A 3 min exposure to a medium-strength vortex revealed a cortisol response that peaked at 6 dpf, marking significant changes in GC_R across early development. Whole-body cortisol levels gradually increased from 2 to 8 dpf, with the strongest vortex-induced elevations observed at 6 dpf. These results not only confirmed the rapid activation of the HPI axis under controlled vortex conditions, consistent with previous reports of elevated cortisol 10 min after brief acute stressors such as fast water movements, mild electric shocks, or air exposure [29,34,64], they also pointed to critical developmental shifts in the HPI axis between 4 and 6 dpf, thus laying the groundwork for further research on HPI axis calibration in response to early-life stress [55]. This follow-up work, which explored the impact of early-life environmental challenge on cortisol regulation, is discussed in more detail below.

While the external development of embryos offers advantages for analysing GR and mineralocorticoid receptor (MR) function during critical periods [26,27], gaps remain in our understanding of post-acute stressor cortisol dynamics in zebrafish larvae. Specifically, the refractory period between stress exposures and the role of GR in early glucocorticoid-negative feedback (GC-NF) remained undefined until recently. Using water vortices, we addressed this gap by examining GC_R dynamics and cortisol feedback regulation in zebrafish larvae [54]. Once again, we confirmed that whole-body cortisol levels peaked 10 min after a 3 min exposure to a medium-strength vortex, returning to baseline within 30–40 min, depending on stressor intensity. Notably, repeated vortex exposures revealed a refractory period of at least 30 min, with cortisol levels rising above baseline only when a second vortex occurred 60 or 120 min after the first. However, cortisol levels did not increase after a 30 min interval, suggesting a rapid cortisol-mediated feedback mechanism regulating GC_R. Furthermore, when larvae were pretreated with the GR antagonist mifepristone,

baseline cortisol levels remained elevated, and the refractory period was suppressed, confirming a GR-dependent feedback process. These findings were in line with previous studies using mifepristone [49,65], which showed prolonged cortisol release in response to repeated stressors, reinforcing the role of GR in modulating GC_R. We also used the defined refractory period in GC_R to investigate the link between early-life challenge and cortisol regulation, as discussed below.

9. Effect of Vortex-Induced Early Life Challenge on Cortisol Response and Behavioural Adaptations

Building on the observed vortex-induced activation of the HPI axis and the identification of critical developmental shifts in GC_R between 4 and 6 dpf under controlled vortex conditions, we employed a high-throughput, prolonged forced swim challenge to investigate the effects of early-life challenge (ELC) on zebrafish larvae [53]. This ELC, implemented at 5 dpf and characterised by sustained rheotaxis over a 9 h period, triggered prolonged HPI axis activation and altered cortisol responses to both homotypic and heterotypic stressors assessed 1–4 days later. At 6 dpf, larvae exposed to vortex flows during ELC showed reduced GC_R, with basal cortisol levels comparable to controls but diminished responses to re-exposure to medium-strength vortices. This attenuation of GC_R persisted through 10 dpf. Furthermore, when exposed to a hyperosmotic medium as a heterotypic stressor, 6 dpf ELC-exposed larvae showed reduced cortisol responses to moderate and high salt stress compared to controls, revealing that the attenuated GC_R was not due to habituation to sensory input [66]. Instead, the sustained reduction likely reflected changes in state variables of the HPI axis. Notably, behavioural adjustments could also be traced back to ELC, including enhanced spontaneous activity, decreased startle reactivity, and improved energy efficiency during rheotaxis. Together, these findings show that vortex-induced ELC can influence both physiological stress responses and active behaviour, revealing intricate adaptations to early-life environmental challenges. The mechanisms driving these effects remain to be elucidated.

10. ELC and HPI Axis Plasticity

Building on the physiological and behavioural correlates of vortex-driven ELC, we further examined its effects on cortisol regulation, neuropeptide expression in the NPO, and gene transcription. This analysis revealed previously unreported changes in cortisol responses at 6 dpf [55].

Zebrafish larvae exposed to ELC showed a distinct cortisol reactivity pattern: diminished cortisol responses to a brief vortex exposure 24 h after ELC, coupled with increased responses to a second vortex within a 30 min refractory period. This pattern suggests adaptive recalibrations in HPI axis signalling and cortisol metabolism, influenced by the timing and sequence of stressors. At 6 dpf, multicolor fluorescent in situ hybridization revealed a reduction in *crh*-, *avp* (arginine vasopressin)-, and *oxt* (oxytocin)-positive cells in the NPO of ELC larvae, along with decreased *crh* and *avp* co-expression [55]. In teleosts, AVP and OXT orthologs are vasotocin (VT) and isotocin (IT), respectively, thought to serve analogous roles in stress regulation, social behaviour, and homeostasis [67–69]. In mammals, these peptides have been well-established: CRH initiates stress responses and influences autonomic and behavioural processes; AVP regulates stress, social behaviour, osmoregulation, and blood pressure; and OXT modulates stress, anxiety, social bonding, and reproduction [70,71]. Conserved functions in stress response and homeostasis are therefore anticipated in zebrafish, and the observed changes suggest that NPO cells show stress-induced plasticity similar to PVN cells in mammals [72–75].

Interestingly, under baseline conditions at 6 dpf, larvae previously exposed to a prolonged vortex at 5 dpf showed fewer *crh*-, *avp*-, and *oxt*-positive cells in the NPO, whereas whole-body qPCR performed 1–2 h after an acute 3 min vortex stress at 6 dpf revealed upregulation of these genes. In other words, while anatomical assessments under resting conditions indicate fewer stress-related neurons in the NPO, functional transcript analysis following acute stress shows enhanced gene expression. It is important to recognise that whole-body qPCR, although useful for detecting overall transcriptional changes, lacks the spatial resolution and specificity of in situ methods; the upregulation may thus include contributions from tissues beyond the NPO. Moreover, differences in the timing and experimental conditions between the two techniques further complicate direct comparisons. Nonetheless, these findings suggest that ELC may induce adaptive changes in stress-related circuitry—resulting in a baseline reduction in key neuropeptide-positive cells while facilitating a heightened transcriptional response upon acute stress.

Complementary whole-body qPCR also revealed upregulation of genes involved in cortisol metabolism and steroidogenesis, consistent with previous research [62]. Specifically, *hsd11b2* (11-hydroxysteroid dehydrogenase type 2), critical for cortisol inactivation [76–78]; *cyp11c1* (11β-hydroxylase), essential for synthesising 11-ketotestosterone and cortisol [79,80]; and *star* (steroidogenic acute regulatory protein), which facilitates cholesterol transport [81]. Additionally, genes related to stress modulation, including *crh*, *avp*, and *oxt*, were also upregulated. Together, these findings suggest that ELC induces HPI axis plasticity, optimising cortisol regulation to balance responsiveness and protection during repeated stress. This is consistent with previous studies showing lasting effects of early-life stress and GC manipulation on HPA/I axis function and stress reactivity [82–84].

Based on these observations, we hypothesise that prolonged early-life activation of the HPI axis, driven by increased environmental challenges, reduces the regulatory capacity of the NPO over downstream HPI activity. Under baseline conditions, this results in enhanced cortisol inactivation, a state that persists during stress exposure. Simultaneously, increased capacity for cortisol synthesis and elevated levels of stress modulators allow for precise control of cortisol dynamics. These adaptations may enable larvae to prevent excessive cortisol release while maintaining a rapid and effective response to familiar stressors [55]. Future studies could leverage zebrafish larvae and vortex protocols as efficient, high-throughput tools to further dissect the molecular and neural mechanisms underlying HPI axis plasticity during early development.

Our findings contribute to a broader understanding of how early-life cortisol dynamics influence neuropeptide expression and GC feedback. Chronic stress in zebrafish larvae has been shown to result in sustained cortisol elevation, increased GC and MR expression, and anxiety-like behaviours later in life [85]. Additionally, optogenetic modulation of interrenal steroidogenic cells using light-sensitive adenylyl cyclase in transgenic zebrafish larvae has shown that persistent cortisol elevation during early development can disrupt neurogenesis, stunt growth, and impair feeding [82,84]. Similar effects have been observed across species, including rodents and humans, where chronic GC exposure or prenatal stress leads to subsequent alterations in stress responsiveness [22–24,86].

In our study [55], ELC larvae showed transcriptional changes suggesting an enhanced balance between cortisol synthesis and inactivation under both baseline and stress conditions. While whole-body qPCR lacks spatial resolution, the upregulation of *hsd11b2* and *cyp11c1* indicates systemic adjustments in cortisol metabolism. In parallel, altered *crh* expression patterns suggest shifts in the maturation of stress-related neural circuits. These findings highlight the NPO as a key mediator of GC feedback and stress adaptation, reinforcing its role as a central regulator of HPI axis plasticity.

11. Future Directions

The integration of larval zebrafish as a model system with high-throughput water vortex protocols has advanced our understanding of HPI axis functionality during early development. This approach has provided insights into the dynamics of cortisol responses, GC regulation, neuropeptide expression in the NPO, and stress-induced transcriptional plasticity, underscoring its utility in dissecting the molecular and physiological mechanisms underlying stress responses. Nonetheless, several key questions warrant further investigation.

To elucidate the broader physiological impacts of vortex-induced stress, future research should further examine the metabolic and cardiorespiratory effects of vortex exposure and compare forced swimming under vortex conditions with routine swimming. Additionally, assessing whole-body lactate and cholesterol levels, cardiac activity, and markers of anaerobic metabolism could provide a more comprehensive understanding of the physiological demands imposed by vortex exposure. These measures would complement current findings, where the cortisol response to vortices reflects significant energy demands and physiological stress associated with these conditions.

Another critical area of investigation involves the mechanisms underlying reduced GC_R to closely spaced stressors. Non-glucocorticoid mechanisms, such as those observed in mice—where repeated stress familiarity reduces CRH neuron activity independently of GC-NF [87]—may also operate in zebrafish and merit further exploration. Additionally, the role of MR alongside GR in regulating the HPI axis remains poorly understood. Future studies should delineate their distinct and synergistic contributions to early GC feedback, investigate age-dependent effects of GR blockade, and evaluate responses to heterotypic stressors. Exploring alternative GR antagonists, as well as utilising GR and/or MR knockout models and assessing GR-responsive gene expression, could further elucidate GC_R dynamics and feedback mechanisms.

Furthermore, the rapid, early phase of GC-NF, mediated by GR within minutes, represents an exciting frontier in stress biology. This phase involves swift GR-mediated inhibition of hormone secretion, enabling dynamic modulation of HPI axis activity in response to glucocorticoid pulses. Emerging evidence suggests that non-genomic mechanisms, such as modulation of membrane excitability and calcium signalling, also play a role in early GC inhibition [88]. Investigating potential crosstalk between genomic and non-genomic GR pathways could uncover novel regulatory mechanisms and refine our understanding of how the HPI axis integrates acute stress signals.

Together, these research directions promise to enhance the utility of the zebrafish model in stress research, offering new perspectives on the interplay between physiological demands, neuroendocrine signalling, and HPI axis plasticity during early development. By addressing these open questions, future studies can build on the foundation established by the combination of high-throughput water vortex protocols and zebrafish larvae, paving the way for a deeper understanding of stress regulation across vertebrates.

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