

## SPECIAL ISSUE REGULAR PAPER

# Olfactorily-mediated cortisol response to chemical alarm cues in zebrafish *Danio rerio*

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Chemical cues released as a by-product of predation mediate antipredator behaviour, but little is known about the physiological responses to olfactory detection of predation risk. In this study, zebrafish *Danio rerio* were exposed to either chemical alarm cues from conspecifics, or water (control). Compared with water controls, *D. rerio* exposed to alarm cues responded behaviourally with antipredator behaviours such as erratic dashing and an increase in time spent near the bottom of the test aquarium. *Danio rerio* were sacrificed 5 min after exposure to test cues (alarm cues or water). Enzyme-linked immunosorbent assay (ELISA) revealed whole-body levels of cortisol that were significantly higher for fish exposed to alarm cues (mean  $\pm$  SE,  $11.9 \pm 3.4$  ng g<sup>-1</sup>) than control fish ( $1.5 \pm 0.7$  ng g<sup>-1</sup>). These data provide a benchmark for future studies of the proximate mechanisms of olfactorily mediated antipredator responses, modelling effects on aquatic life in a changing climate and, as a model organism, *Danio rerio* can further our understanding of anxiety in humans.

## KEYWORDS

chemical alarm cues, cortisol, *Danio rerio*

## 1 | INTRODUCTION

In aquatic ecosystems, chemical cues mediate predator–prey interactions, informing prey about the presence of predation risk, the identity and diet of predators and a rich array of variables correlated with risk that prey learn and use to avoid future exposure to predation risk (Ferrari *et al.*, 2010; Kelley & Magurran, 2003; Wisenden, 2015). When a predator attacks a fish, chemical alarm cues released from damaged epidermal tissue reliably indicate the presence of an actively foraging predator. Exposure to alarm cues elicits behavioural responses such as dashing (erratic swimming), increased shoal cohesion, fin flicking, increased use of shelter, increased opercular pumping and other outward signs of distress and anxiety (Chivers & Smith, 1998; Egan *et al.*, 2009; Ferrari *et al.*, 2010; Mathuru *et al.*, 2012; Speedie & Gerlai, 2008) and information gathering (Sutrisno *et al.*, 2014; Wisenden *et al.*, 2010).

The physiological effects of exposure to chemical alarm cues have received far less attention. Cortisol is a ubiquitous steroid hormone that is commonly associated with stress and easily measured in fish (Johnstone *et al.*, 2012). Stress increases cortisol levels, which affects the regulation of metabolic processes and immune function (Barton &

Iwama, 1991; Mommsen *et al.*, 1999). These physiological effects help prepare fish for behavioural changes involved in fight-or-flight responses (Egan *et al.*, 2009; Johnstone *et al.*, 2012).

Perception of predation risk *via* chemical alarm cues increases plasma or whole-body cortisol in a range of fish species. Cortisol has been shown to increase in response to exposure to predators (Barcellos *et al.*, 2007), dead conspecifics (Oliveira *et al.*, 2014) and by physical handling in aquaculture settings (Barton & Iwama, 1991; Ramsay *et al.*, 2009).

In this study, we quantify the increase in whole-body cortisol levels in zebrafish *Danio rerio* (Hamilton 1822) that occurs when they are exposed to chemical alarm cues derived from the skin of conspecifics. These alarm cues cause behavioural responses in *D. rerio* (Hall & Suboski, 1995; Korpi & Wisenden, 2001; Mathuru *et al.*, 2012; Speedie & Gerlai, 2008; Suboski *et al.*, 1990; Wisenden, 2011), therefore we anticipate a cortisol response as well. Confirmation of this would further illuminate the role of cortisol in the regulation of risk assessment and predator avoidance, which will set the stage for future studies in physiological–ecological interactions and how these interactions may be affected by anthropogenic changes in aquatic environments.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental apparatus and cue preparation

We acquired wild-type adult *Danio rerio* about 3–4 months of age from a commercial breeder and maintained laboratory stocks in bare glass aquaria (75 l) on a diet of commercial flake food alternated with brine shrimp nauplii and maintained on a 12L:12D light cycle. We prepared alarm cue by euthanizing 22 adult fish (mean  $\pm$  1 SE total length,  $L_T = 34.5 \pm 0.55$  mm), discarding the heads and homogenizing the bodies in 100 ml of dechlorinated tap water. The resulting solution was filtered through a loose wad of polyester fibre, diluted to 220 ml and aliquoted into 10 ml doses and frozen at  $-20^\circ\text{C}$  until needed. Alarm-cue concentration represented one donor fish per trial. We also prepared 10 ml doses of blank dechlorinated tap water for the control treatment and froze them at  $-20^\circ\text{C}$  until needed.

Standard glass aquaria (37 l) were filled with dechlorinated tap water, heated to  $25^\circ\text{C}$  and fitted with a large sponge filter. An additional airline tube was inserted into the exit tube of the sponge filter. The other end of the extra airline tube exited the tank and was affixed to the shelf supporting the aquarium, leaving c. 1 m of tubing to allow the observer to surreptitiously inject test stimuli into the test tank. Turbulent water and air currents created by the filter masked minor pressure gradients created by cue injection and quickly dispersed test cues throughout the tank (less than 15 s in dye tests; Wisenden, 2011). A grid with cells  $5 \times 5$  cm was drawn on the front pane of each aquarium that allowed an observer to quantify activity and vertical distribution. Two fish were placed in each tank and allowed to acclimate for at least 12 h.

### 2.2 | Behavioural protocol

Fish were observed by a calm observer positioned about 1 m from the tank. Room lights were dimmed to reduce the effect, if any, of observer presence. Activity, measured as the number of grid lines crossed by both fish, was tallied over 5 min immediately prior to stimulus injection. We simultaneously recorded vertical distribution at 15 s intervals as the horizontal row in the grid occupied by each fish. After 5 min we gently introduced 10 ml of thawed test cue (either alarm cue or water) through the injection tube and flushed the injection tube with 60 ml of previously-retained tank water. Cue injection required about 60 s to complete, by the end of which test cues are dispersed throughout the aquarium. Once cue injection was completed, we immediately began another 5 min observation of activity and vertical distribution as described above. The fish were then immediately removed, euthanized by an overdose of MS-222, flash frozen by immersion in liquid nitrogen and then stored in a labelled vial at  $-80^\circ\text{C}$ . We ran eight trials using water as the test cue (control) and eight trials using alarm cue as the test cue.

### 2.3 | Cortisol extraction and measurement

Whole-body cortisol extraction was adapted from the method described by Canavello *et al.* (2011). Individual fish were weighed, partially thawed to allow efficient mincing, then homogenized in 1 ml of ice-cold phosphate buffered saline (PBS) using a Cole-Parmer LabGen

125 homogenizer ([www.coleparmer.co.uk](http://www.coleparmer.co.uk)) in  $5 \times 15$  s bursts followed by homogenizer wash with an additional  $2 \times 1$  ml PBS. The homogenate and washes were combined and extracted with 5, 3 ml volumes of diethyl ether. The resulting solution was vortex-mixed for 1 min then centrifuged at 2075 g for 5 min to separate aqueous and ether layers. The upper ether layers containing cortisol were removed and combined. Ether extracts were evaporated in a fume hood overnight to near dryness. Final ether removal was achieved under nitrogen. Samples from individual fish were reconstituted with 1 ml PBS and reconstituted for 24 h. All steps were carried out at  $4^\circ\text{C}$ . Enzyme-linked immunosorbent assay (competitive ELISA) for human salivary cortisol (Salimetrics; [www.salimetrics.com](http://www.salimetrics.com)) modified for whole body *D. rerio* (Canavello *et al.*, 2011) was used to quantify cortisol. Absorbances from reactions were measured at 450 nm using a BioTek Synergy HTX plate reader ([www.biotek.com](http://www.biotek.com)). Net absorbances were determined by subtracting non-specific binding values. Cortisol concentrations in 0.1 ml samples were determined by comparison to  $BB_0^{-1}$  (*i.e.*, net sample A450 per zero control A450) v. cortisol ( $0\text{--}3.0 \mu\text{g dl}^{-1}$ ). All samples were assayed in quadruplicate (two different dilutions, each in duplicate) and resultant cortisol levels were normalized based on fish mass and expressed as ng cortisol  $\text{g}^{-1}$  fish mass.

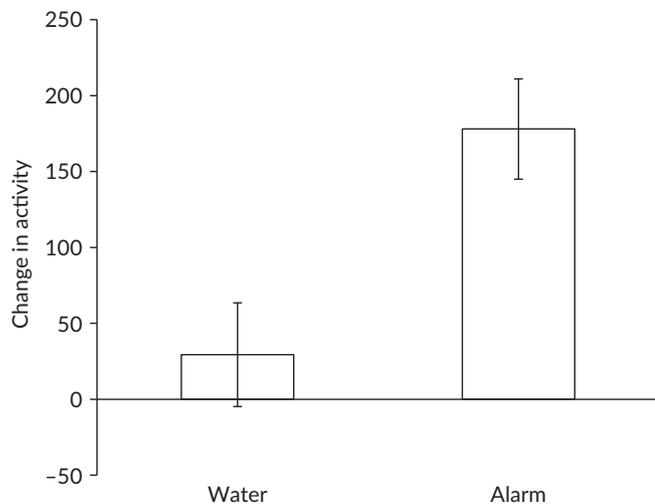
The methods used for this research were reviewed and approved by the Minnesota State University Moorhead Animal Care and Use Committee under protocol 16-R/T/BIO-018-N-Y-C.

### 2.4 | Data analysis

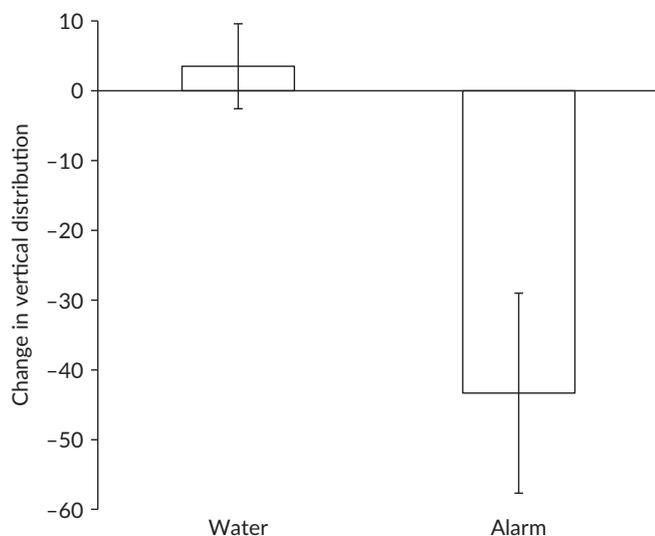
Change in activity and vertical distribution for each trial were calculated by subtracting the sum score of the values for the two test fish in the pre-stimulus period from the sum score of their values in the post-stimulus period. Kilmogorov-Smirnov tests showed that change in activity and change in vertical distribution were normally distributed (activity:  $z = 0.829$ ,  $P > 0.05$ , vertical distribution:  $z = 0.999$ ,  $P > 0.05$ ), therefore a Student's *t*-test was used to compare treatment groups. Technical replicates of cortisol measures for each fish were averaged to create a single datum per fish. Cortisol measures were analysed with nested ANOVA to control for lack of statistical independence between fish pairs within each tank.

## 3 | RESULTS

*Danio rerio* responded to alarm cues with an alarm reaction that involved change in activity and movement to the bottom. Change in activity was significantly greater for trials that received alarm cues than for trials that received water ( $t_{14} = 3.127$ ,  $P < 0.01$ ; Figure 1). The change in activity was significantly different from 0 for alarm cue trials (one-sample *t*-test  $t_7 = 5.391$ ,  $P < 0.001$ ) but not for water trials ( $t_7 = 0.859$ ,  $P > 0.05$ ). The change in vertical distribution was significantly lower for trials that received alarm cues than for trials that received water ( $t_{14} = 3.024$ ,  $P < 0.01$ ; Figure 2). The change in vertical distribution was significantly different from 0 for alarm cue trials ( $t_7 = 3.042$ ,  $P < 0.05$ ) but not for water trials ( $t_7 = 0.574$ ,  $P > 0.05$ ). Cortisol levels were  $11.91 \text{ ng g}^{-1}$  fish mass on average for alarmed fish and



**FIGURE 1** Mean ( $\pm$  SE) change in activity (number of grid lines crossed) for pairs of *Danio rerio* exposed to water or conspecific alarm cues ( $t_{14} = 3.127$ ,  $P < 0.01$ )

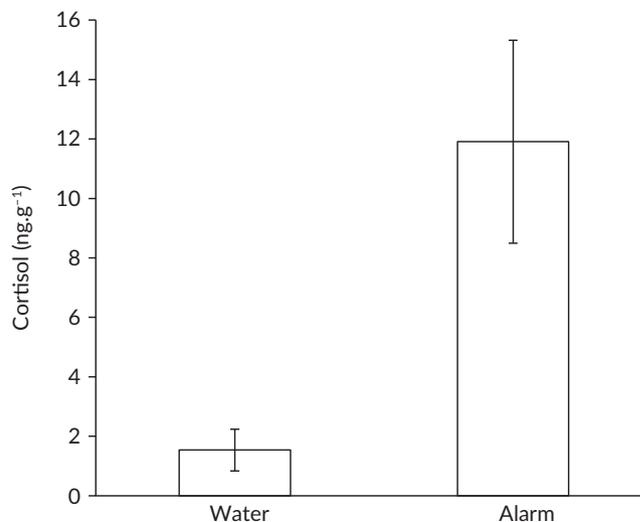


**FIGURE 2** Mean ( $\pm$  SE) change in vertical distribution (horizontal rows on the grid) for pairs of *Danio rerio* exposed to water or conspecific alarm cues ( $t_{14} = 3.024$ ,  $P < 0.01$ )

1.538 ng g<sup>-1</sup> fish mass for control fish, which represents a 7.9-fold increase ( $F_{1,14} = 5.77$ ,  $P < 0.05$ ; Figure 3).

#### 4 | DISCUSSION

The behavioural response to alarm cues conformed to known components of alarm reactions (Ferrari *et al.*, 2010; Mathuru *et al.*, 2012; Speedie & Gerlai, 2008). *Danio rerio* frightened by alarm cue engaged in erratic dashing that increased overall activity and they also spent more time near the bottom of the tank. These behavioural changes result in reduced probability of predation in fathead minnows *Pimephales promelas* Rafinesque 1820 (Mathis & Smith, 1993), brook charr *Salvelinus fontinalis* (Mitchill 1814) (Mirza & Chivers, 2002), rainbow trout *Onchorhynchus mykiss* (Walbaum 1792) (Mirza & Chivers, 2003), amphipods *Gammarus minus* (Wisenden *et al.*, 1999) and toad *Bufo*

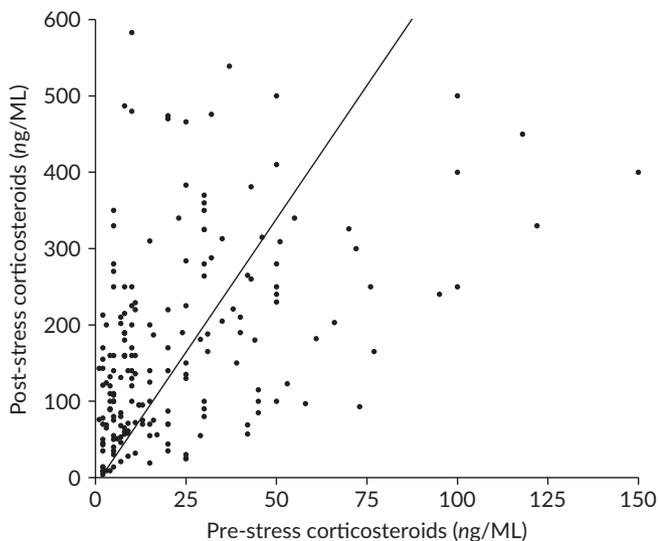


**FIGURE 3** Mean  $\pm$  1SE whole-body cortisol for zebrafish *Danio rerio* Hamilton 1822 exposed to water or conspecific alarm cues ( $F_{1,14} = 5.77$ ,  $P = 0.031$ )

*boreas* tadpoles (Hews, 1988). The behavioural data confirm that fish that received alarm cues were in a frightened or stressed state when they were sampled for cortisol analysis. Alarmed *Danio rerio* had whole-body cortisol levels that were 7.9 times higher than *Danio rerio* exposed to water control cue.

The active compounds in conspecific alarm cue have been the focus of an extensive literature but the biochemistry is still not well understood. Early work hypothesized that an alarm cue was produced in specialized club cells in the epidermis but more recent work suggests that this may not be the case (Carreau-Green *et al.*, 2008; Chivers *et al.*, 2007). Active compounds in skin extract probably include hypoxanthine 3(N) oxide (Brown *et al.* 2000, 2001, 2003; Parra *et al.*, 2009), but empirical support for hypoxanthine 3(N) oxide as the sole active ingredient has not survived careful scrutiny (Ferrari *et al.*, 2010; Mathuru *et al.*, 2012; Wisenden, 2015). There is biochemical evidence that chondroitin sulphate is a component of alarm cue in *Danio rerio* (Mathuru *et al.*, 2012), northern studfish *Fundulus catenatus* (Storer 1846) (Farnsley *et al.*, 2016) and *P. promelas* (Faulkner *et al.*, 2017), but, similar to hypoxanthine 3(N) oxide, chondroitin sulphate does not confer full potency relative to raw-skin extract and also lacks species specificity. Alarm cue is most likely to be a mixture of compounds that confer some phylogenetically inherited components and other components that are species-specific (Wisenden, 2015).

The magnitude of the cortisol response found in our study is difficult to compare directly with the magnitude of responses in other studies because of differences among species and differences in methods used by various authors to elicit a cortisol response and to quantify cortisol. In the current study, whole-body cortisol increased from 1.5 to 11.9 ng g<sup>-1</sup>, an almost eightfold increase in response to chemical alarm cues. This is similar in magnitude, but not in terms of absolute values, to the change in whole-body cortisol in *D. rerio* exposed to the odour of dead conspecifics (control ~ 10 ng g<sup>-1</sup> v. ~ 70 ng g<sup>-1</sup>) (Oliveira *et al.*, 2014). Plasma cortisol levels doubled from ~10 ng ml<sup>-1</sup> in control to ~20 ng ml<sup>-1</sup> in frillfin gobies *Bathygobius soporator* (Valenciennes 1837) exposed to chemical alarm cues



**FIGURE 4** Change in corticosteroids before versus after subjected to various forms of handling stress across a variety of aquacultured fish species. The overall relationship between Post and Pre-stress corticosteroids was  $\text{Post} = 6.99 \cdot \text{Pre} - 10.43$ ,  $F_{1,193} = 104.23$ ,  $P < 0.001$ ,  $R^2_{(\text{adj})} = 0.347$ . Fish species include those from the following families: Polyodontidae, Lepisosteidae, Amiidae, Anguillidae, Clupeidae, Salmonidae, Cyprinidae, Catostomidae, Ictaluridae, Cyprinodontidae, Perichthyidae, Centrarchidae, Percidae, Sciaenidae, Scombridae, and Pleuronectidae. Data presented here were extracted from Table 1 of the review by Barton & Iwama (1991)

(Barreto *et al.*, 2014) and doubled from  $\sim 28 \text{ ng ml}^{-1}$  to  $\sim 57 \text{ ng ml}^{-1}$  in Nile tilapia *Oreochromis niloticus* (L. 1758) exposed to conspecific alarm cues (Sanches *et al.*, 2015).

Visual presentation of a predator or physical interaction with a predator increased whole body cortisol levels from a baseline of  $\sim 7 \text{ ng g}^{-1}$  in control to  $\sim 9\text{--}13 \text{ ng g}^{-1}$  in experimental fish (Rehnberg *et al.*, 1987) and by only a factor of 2.5 from  $\sim 6 \text{ ng g}^{-1}$  in controls to  $\sim 15.5 \text{ ng g}^{-1}$  in *D. rerio* exposed to a predator (Barcellos *et al.*, 2010). Physical contact and handling also causes a cortisol response. Netting *D. rerio* and suspending them in the air for 3 min, twice, induced increased whole-body cortisol threefold from a baseline of  $\sim 10 \text{ ng g}^{-1}$  to  $\sim 28 \text{ ng g}^{-1}$  (Ramsay *et al.*, 2009), whereas treatment with anxiety-reducing fluoxetine reduced (sevenfold) whole-body cortisol from  $0.07 \text{ ng g}^{-1}$  in control to  $0.01 \text{ ng g}^{-1}$  (Egan *et al.*, 2009). Handling stress in aquaculture fish species has been studied for many years (Barton & Iwama, 1991). A meta-analysis of 195 studies in this literature (Barton & Iwama, 1991) revealed an average of a sevenfold increase in corticosteroids in post v. pre-stress levels across a broad range of fish taxa, stressors and analytic methods (Figure 4). Our data showing an eightfold increase in cortisol in response to chemical alarm cues are consistent with this broad meta-analysis of the effect of stress on cortisol levels in fishes.

#### 4.1 | Future research directions

These data open new possibilities for multiple areas of future study. The first is that cortisol can be used as new tool for measuring the detection of odorants related to predation. Electro-olfactograms

(EOG) record the activation of olfactory receptors by semiochemicals, but they do not provide information about the cognitive processing of this information. Overt behavioural responses to alarm cues such as area avoidance, seeking shelter, *etc.*, clearly indicate detection of chemical information and cognitive interpretation and decision to initiate antipredator behaviour. However, chemical information about risk does not always elicit an overt behavioural response. Cognitive recognition of elevation of ambient risk would call for a general elevation of cortisol to prepare physiologically for attack evasion.

Anthropogenic degradation of aquatic habitats is widespread but the effects on aquatic life are not well understood. For example, we are only beginning to understand the sublethal effects of heavy metal contamination of freshwater environments (Dew *et al.*, 2014) or ocean acidification due to global changes in atmospheric  $\text{CO}_2$  (Ferrari *et al.*, 2011). Under these conditions, we predict that cortisol-mediated changes in metabolism (Mommensen *et al.*, 1999) and maternal effects (Sloman, 2010; Sopinka *et al.*, 2017) would be affected.

The ecological role of detection and response to chemical alarm cues is relatively well-studied (Ferrari *et al.*, 2010), but how ecological interactions regulate internal physiological processes and how internal cortisol-regulated processes in turn influence ecological interactions is ripe for exploration. Because cortisol levels can be experimentally manipulated using alarm cues, new lines of research could explore ways that olfactory perception of predation risk affect physiological processes after short-term and chronic exposure to risk (Archard *et al.*, 2012). Previous studies have shown that behavioural response intensity is positively correlated with concentration of alarm cues (Brown *et al.*, 2006; Hawkins *et al.*, 2007; Lönnstedt & McCormick, 2011; Mirza *et al.*, 2006; Mirza & Chivers, 2003). We might predict that magnitude of the cortisol response might also covary with intensity of behavioural response. Olfactory detection of sex pheromones can also cause elevation of cortisol in *D. rerio* (Reolon *et al.*, 2018), indicating that olfactorily-mediated cortisol responses are not limited to the context of predator-prey interactions. Distressed, but uninjured fish release disturbance cues that alert nearby conspecifics of the presence of danger. Disturbance cues are probably derived from metabolic by-products (Vavrek *et al.*, 2008), or from individuals that are nutritionally-stressed (Abreu *et al.*, 2016) or parasitized (Triki *et al.*, 2016), all of which suggest that circulating cortisol levels could be used by conspecifics to guide behavioural interactions in a variety of ecological contexts.

Finally, *D. rerio* are a commonly used model organism for studying stress and anxiety in humans because the physiological mechanisms of stress are conserved across the evolutionary trajectory of vertebrates (Egan *et al.*, 2009; Kalueff *et al.*, 2012; Speedie & Gerlai, 2008; Steenbergen *et al.*, 2011). The adrenal glands of humans are homologous to inter-renal tissue in the head kidney in fish and amphibians (Harris & Carr, 2016). Activation of the hypothalamus-pituitary-inter-renal axis releases corticotropin, adrenocorticotrophic hormone and cortisol (Egan *et al.* 2009). In this regard, *D. rerio* are a good model species for studying human stress responses as both *D. rerio* and humans use cortisol as their primary stress hormone, whereas rodents use corticosterone (Egan *et al.* 2009). The data reported here demonstrate that alarm cue can be used as a reliable tool to produce elevated levels of cortisol in *D. rerio*.

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