Thermal stress induces a positive phenotypic and molecular feedback loop in zebrafish embryos

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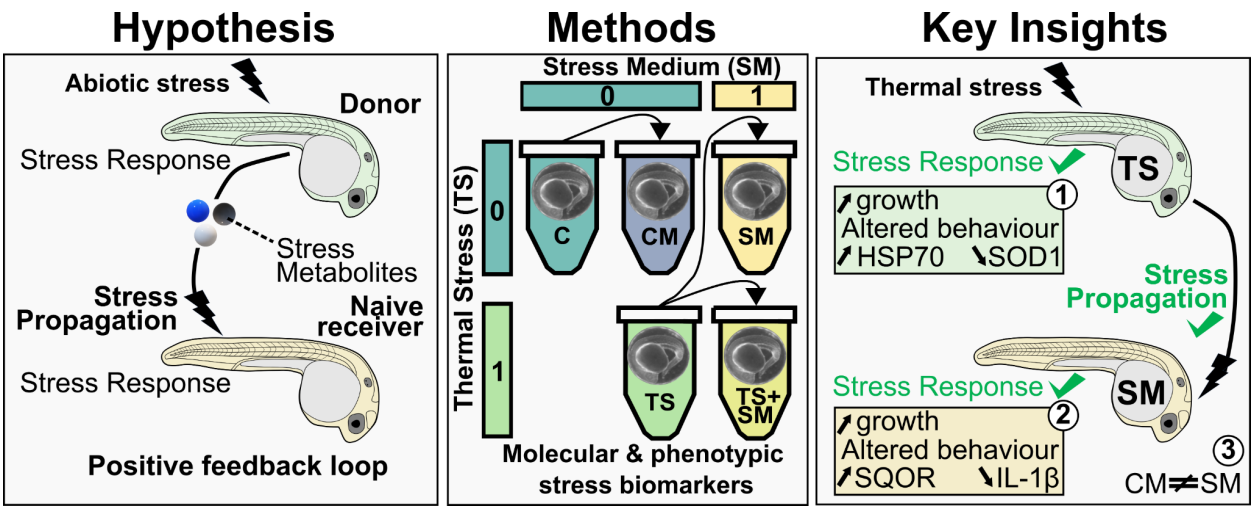
Graphical abstract



Highlights

- Repeated sublethal thermal stress induces a stress response in zebrafish embryos.
- Stressed embryos chemically signal to naive conspecifics via the medium.
- Directly and indirectly stressed embryos show similar behaviour and faster growth.
- Stress medium receivers have altered immune and antioxidant gene expression.

Abstract

Aquatic organisms must cope with both rising and rapidly changing temperatures. These thermal changes can affect numerous traits, from molecular to ecological scales. Biotic stressors are already known to induce the release of chemical cues which trigger behavioural responses in other individuals. In this study, we infer whether fluctuating temperature, as an abiotic stressor, may similarly induce stress-like responses in individuals not directly exposed to the stressor. To test this hypothesis, zebrafish (*Danio rerio*) embryos were exposed for 24 hours to fluctuating thermal stress, to medium in which another embryo was thermally stressed before ("stress medium"), and to a combination of these. Growth, behaviour, expression of molecular markers, and of whole-embryo cortisol were used to characterise the thermal stress response and its propagation between embryos. Both fluctuating high temperature and stress medium significantly accelerated development, by shifting stressed embryos from segmentation to pharyngula stages, and altered embryonic activity. Importantly, we found that the expression of sulfide:quinone oxidoreductase (SQOR), the antioxidant gene SOD1, and of interleukin-1β (IL-1β) were significantly altered by stress medium. This study illustrates the existence of positive thermal stress feedback loops in zebrafish embryos where heat stress can induce stress-like responses in conspecifics, but which might operate via different molecular pathways. If similar effects also occur under less severe heat stress regimes, this mechanism may be relevant in natural settings as well.

Keywords: behaviour, chemical communication, heat stress, gene expression, feedback loop, zebrafish embryos

1. Introduction

Fish are ectothermic vertebrates susceptible to changes in the thermal environment, particularly to higher temperatures close to their upper thermal limits (Araújo et al., 2013; Morgan et al., 2001; Paaijmans et al., 2013). Early developmental stages have narrower thermal ranges than adults (Skjærven et al., 2014). Temperature regimes during development have irreversible effects as they modulate subsequent stages. Examples of persistent effects of temperature changes during development include alterations of swimming performance and cardiac anatomy (Dimitriadi et al., 2018), masculinisation (Ribas et al., 2017) and increased mortality (Hosseini et al., 2019). Together these can shape the future trajectory of populations, as global warming is characterised by both greater thermal variability and more extreme thermal events of longer duration and magnitude (Pörtner et al., 2019; Vasseur et al., 2014).
The zebrafish (*Danio rerio*) is emerging as a model organism to study the effects of thermal stress (Brown et al., 2015; Clark et al., 2011; Long et al., 2012; Luu et al., 2020). Adult zebrafish are eurythermal, naturally tolerating a wide range of temperatures (16.5-38.6°C) with optimal temperature around 27-28.5°C. They may face natural thermal variations of ~5°C daily, and from 6 to 38°C seasonally (Engeszer et al., 2007; López-Olmeda and Sánchez-Vázquez, 2011; Spence et al., 2008). However, early stages of zebrafish only tolerate minimum temperatures of 22-23°C and maximum temperatures of around 32°C to develop normally (Pype et al., 2015; Schirone and Gross, 1968; Schnurr et al., 2014). These warm-adapted fish that are living near their upper thermal limit may be among the "losers" of climate change (Somero, 2010). Of note, the thermal biology of zebrafish is conserved in laboratory populations, in spite of laboratory domestication, which makes them an adequate model organism to investigate the effects of thermal challenges in the laboratory (Brown et al., 2015; Morgan et al., 2019).
Heat-stressed zebrafish larvae display anxiety-like behaviours (Bai et al., 2016), transient hyperactivity (Yokogawa et al., 2014) and altered embryonic development (Hallare et al., 2005; Hall and Warner, 2021; Long et al., 2012; Schröter et al., 2008). At the molecular level, heat stress leads to accumulation of reactive oxygen species (Madeira et al., 2016; Vinagre et al., 2012), and to changes in both global gene expression patterns (Logan and Buckley, 2015; Long et al., 2012; Ribas et al., 2017) and in the hypothalamic-pituitary-interrenal (HPI) axis (Alsop and Vijayan, 2009). For example, the expression of Cu/Zn-superoxide dismutase I (SOD1) and sulfide:quinone oxidoreductase (SQOR) is modulated by temperature and hypoxia in zebrafish embryos (Icoglu Aksakal and Ciltas, 2018; Long et al., 2015, 2012). The innate immune system is also challenged by both high temperature and thermal fluctuations in teleost early stages (Mariana and Badr, 2019; Zhang et al., 2018). For instance, interleukin-1β (IL-1β), a gene central to the stress response (Goshen and Yirmiya, 2009; Metz et al., 2006; Vitkovic et al., 2000) is upregulated in zebrafish embryos exposed to high temperature (Icoglu Aksakal and Ciltas, 2018).
Largely overlooked to date, however, is the question of whether the response to thermal stress can be transmitted to other individuals. Fish use chemical communication to alert others of a threat using so-called "alarm cues" (released after skin damage) or "disturbance cues" (released without injury following a biotic stressor, (Jordão and Volpato, 2000). Exposure to conspecific predation-related disturbance cues induces stress-like responses in fish (Barcellos et al., 2014, 2011; Bett et al., 2016; Ferrari et al., 2008; Jordão and Volpato, 2000; Toa et al., 2004), including zebrafish (Barcellos et al., 2014). Importantly, even fish at early stages are capable of such chemical communication as they respond to alarm cues (Atherton and McCormick, 2017, 2015; Oulton et al., 2013; Poisson et al., 2017). Typically, stress induces the release of metabolites into the environment, including hormones of the HPI axis such as cortisol (Barcellos et al., 2014; McGlashan et al., 2012), CO_2 (McGlashan et al., 2012), respiratory byproducts, catecholamines, or nitrogenous metabolic products such as urea (Bairos-Novak et al., 2017; Giaquinto and Hoffmann, 2012; Henderson et al., 2017; Hubbard et al., 2003), which might serve as the carrier molecules of these cues.

Despite the wealth of information on such biotic stress-induced stress propagation, this phenomenon is only known for a few abiotic stressors, such as low pH or acute fasting (Abreu et al., 2016; Feugere et al., 2021). Evidence of transmission of heat-induced stress between conspecifics comes mainly from a single experiment in crayfish (Hazlett, 1985). In this contribution, we test the hypothesis that thermal stress

induces cues conferring signals of thermal stress to naive receivers, in which they elicit a response (Fig. 1a). As alarm and disturbance cues have been defined with respect to biotic stressors, we introduce the term "stress metabolite" referring to such cues putatively induced by abiotic stress (Feugere et al., 2021). To test our hypothesis, zebrafish embryos were exposed to independent and combined treatments of thermal stress and medium putatively containing stress metabolites induced by thermal stress. We exposed zebrafish embryos to thirteen heat peaks of +5°C within the 24 hours of the segmentation period. This thermal regime is intended to elicit a high stress response in zebrafish embryos. Zebrafish embryo behaviour, growth rate, and expression of genes involved in the immune response (IL-1β) and antioxidant pathways (SOD1, SQOR) were investigated, alongside an assessment of heat stress-induced HSP70 and the levels of whole-embryo cortisol. Our three main expectations are that (i) fluctuating high temperatures, similar to constant higher temperatures, trigger developmental, behavioural and molecular stress responses in zebrafish embryos, and that (ii) these responses can be propagated to naive receiver embryos through stress medium, which are however (iii) not elicited by fresh or control medium.

2. Materials and Methods

2.1. Experimental design

For detailed zebrafish husbandry and breeding methods, see Supplementary Information. Just before the beginning of experimental treatments, several zebrafish embryos per clutch were photographed to estimate the median initial stage. Exposure began around the blastula stage (median stage: 2.75 hpf, at 512-cells). Fertilised healthy embryos (with chorion) were selected and individually placed into 0.2 mL 8-strip PCR tubes prefilled with 200 µL of 1X E3 medium (Cold Spring Harbor Laboratory Press, 2011).

In a two-factorial design, embryos were exposed to different combinations of the two factors, temperature stress (TS) and used medium conditioned with putative stress metabolites. We use the term "stress medium" (SM) to describe this thermal stress-conditioned medium. We reasoned that any observed effects of stress medium may be due to regularly excreted metabolites, which could accumulate towards the end of each treatment independently of thermal stress and would be more concentrated in SM and combined TS+SM treatments. This warranted the use of an additional control, the "control medium" (CM), that is, medium that had previously hosted control embryos and containing putative regular metabolites in the absence of heat stress, to see whether CM could produce similar outcomes as TS or SM. Embryos were exposed to either thermal stress or control temperature protocols within a thermocycler, incubated in fresh, control, or stress medium. All experimental treatments are detailed in Fig. 1b and Table S1. The thermal stress protocol was designed to maximise potential effects and spanned 16.25 hrs, divided into thirteen 75-min series of temperature fluctuations between 27, 29, 32, 29, and 27°C, with each temperature step being maintained for 15 min. Thermal stress mimicked +5°C temperature peaks over zebrafish optimal temperature (a total of n = 13 peaks) reaching the sublethal temperature of 32°C (Scott and Johnston, 2012).While not replicating realistic conditions of heatwaves, the choice of this thermal regime aimed to elicit maximal responses to repeated exposure to sublethal stress in early embryos at the crucial time of somitogenesis. A recovery period of 7.75 hrs at 27°C followed the fluctuating temperature period to reach a total incubation time of 24 hrs. The control thermal protocol was a steady 27°C for 24 hrs. E3 media following control or thermal stress conditions were reused for "control medium" and "stress medium" treatments, respectively. In total this yielded five treatments: control (C, control protocol with fresh medium), control medium (CM, control protocol with reused medium from C), thermal stress (TS, thermal stress protocol with fresh medium), stress medium (SM, control protocol with reused medium from TS), and thermal stress + stress medium (TS+SM, thermal stress protocol with reused medium from TS). Used media were immediately re-used for treatments containing putative stress or control metabolites, respectively. As development in zebrafish accelerates with higher temperature (Kimmel et al., 1995), but decelerates with darkness (Bucking et al., 2013; Villamizar et al., 2014) additional control embryos were monitored after longer incubation

times: 31 hrs (C31), 37 hrs (C37), and 46 hrs (C46). These times were adjusted for darkness-raised embryos to reach the stages of prim-6 (25 hpf), prim-16 (31 hpf), and late pharyngula (35 - 42 hpf), respectively. Initial, final, and total exposure times were used to standardise each procedure. After incubation, transparent embryos were deemed alive and kept for subsequent steps. Before and after each exposure, embryo media were sampled to measure pH and O_2 saturation levels that could impact embryos in the used media. Oxygen levels were measured using Unisense electrodes (see Supplementary Methods). Constant medium pHs were verified using the Fisherbrand™ accumet™ AB150 pH Benchtop Meters.

2.2. Analysis of phenotypic data

Phenotypic analyses were conducted for all eight treatments. Embryos were placed in a watch glass vial and videoed by small batches using the camera setup (see Supplementary Information) for 20 seconds. Embryos were placed under light (similar intensity across measurements) to elicit a startle-like response after exposure in darkness. Behavioural data was analysed using Danioscope (Noldus). When possible, several videos were recorded for each embryo clutch and behavioural measurements were averaged for each individual embryo. Analysis of behavioural responses were conducted on the video-length standardised burst activity percentage (percentage of the time - from the total measurement duration - the embryo was moving). Final embryonic stages were estimated following Kimmel et al. (1995) from several photographs of embryos within their chorions using the criteria somite number, yolk extension to yolk ball ratio, presence and morphology of otoliths, tail aspect, presence of lens primordium, presence and position of the cerebellum relatively to the eyes, and pigmentation. The growth index was calculated as

$$\Delta hpf/hr = (embryo\ final\ stage\ (hpf) - batch\ median\ initial\ stage\ (hpf))/ batch\ exposure\ time\ (hr).$$

Statistical analyses were conducted in RStudio (RStudio Team, 2020). Outliers among behavioural values were excluded from statistical analyses using Tukey's method with a 1.5 interquartile range cut-off. First, the effects of stress medium and thermal stress predictors across the C, TS, SM, and TS+SM treatments were evaluated. Shapiro-Wilk and Levene's tests were used to evaluate normality and homogeneity of variances, respectively. Normalisation methods were compared using the *BestNormalize* R package (Peterson and Cavanaugh, 2020). The growth index was parameterised using an order normalising transformation (Shapiro-Wilk's P = 0.98, Levene's P = 0.27) and analysed by a two-way ANOVA with thermal stress and stress medium as predictors, followed by *post-hoc* pairwise Student's t-tests between control C and each experimental condition. The burst activity percentage and final stage (in hpf) could not be normalized and were analysed for the effects of both predictors using nonparametric Scheirer-Ray-Hare tests from the *rcompanion* R package (Mangiafico, 2018), followed by pairwise *post-hoc* Wilcoxon-Mann-Whitney tests. The final embryonic developmental period was coded as *segmentation* or *pharyngula* and analysed for the effects of thermal stress, stress medium as factors and initial stages as covariate using a generalised logistic model followed by pairwise *post-hoc* comparisons using the *emmeans* R package (Lenth, 2021).

Additional pairwise comparisons were performed for all response variables of CM against C and SM. To determine whether behavioural effects of treatment were related to developmental acceleration, pairwise comparisons of behaviour of control embryos (C) were compared to that of older control embryos (C31, C37, C46) using Kruskal-Wallis tests. Fourth, burst activity percentages of embryos from stress medium and thermal stress treatments were compared against that of control embryos incubated for longer times using pairwise Wilcoxon-Mann-Whitney tests. Multiple comparisons were corrected using Bonferroni adjustments. Cohen's |d| were obtained from the distribution used to compute the statistical analyses and calculated using the *effsize* r package (Torchiano, 2016) or according to (Lenhard and Lenhard, 2016), respectively. Effect size was qualified based on thresholds given in (Sawilowsky, 2009): very small: $|d| > 0.01$, small: $|d| > 0.2$, medium: $|d| > 0.5$, large: $|d| > 0.8$, very large: $|d| > 1.20$, huge: $|d| > 2.0$.

2.3. Molecular analysis

Gene expression analyses were conducted for CM, C, SM, TS, and TS+SM treatments (n = 3 replicate pools each representing the average gene expression of 60 embryos per treatment i.e. a total of 180 embryos per treatment). Embryos were snap-frozen at -80°C immediately after experimental treatments. Total RNA was extracted using a High Pure RNA isolation kit (Sigma-Aldrich) following the manufacturer's recommendations. cDNA was synthesized using Superscript II^{TM} Reverse Transcriptase (Invitrogen, Life Technologies Ltd.) with sample randomisation. TaqMan® Gene Expression Assays (ThermoFisher Scientific) and 2X qPCR Bio Probe Hi-ROX (PCRBiosystems) were used to quantify the expression of three genes of interest (SQOR, SOD1, and IL-1β) normalised to two reference genes (ef1-α, β-actin). The effects of stress medium and thermal stress on the $Log_2\ 2^{-\Delta\Delta CT}$ (Log_2 fold-change) values were investigated using the *eBayes* and *lmFit* functions within the *limma* package (Ritchie et al., 2015) within the *Bioconductor v.3.11* (Ihaka and Gentleman, 1996) R environment. Next, pairwise *post-hoc* comparisons on C *versus* SM, TS, or TS+SM, and CM *versus* C or CM were performed using pairwise moderated t-tests with Bonferroni adjustments. Effect sizes (Cohen's |d|) were calculated as above. More details on the gene expression analysis are given as Supplementary Information. All data and R code used in this experiment are available as Supplementary Material. To verify that our treatment induces heat stress in embryos, we quantified the protein level of two common heat stress biomarkers, heat shock protein 70 and whole-embryo cortisol levels (see Supplementary Information).

3. Results

3.1. Phenotypic effects of thermal stress and its propagation

First, the phenotypic effects of fluctuating thermal stress and of stress medium treatments were analysed. Embryonic growth indices were significantly accelerated by the factors stress medium (small effect size, $F = 6.291$, $P = 0.0128$), thermal stress (large effect size, $F = 75.502$, $P < 0.0001$), and their combination (very large effect size, $F = 7.498$, $P = 0.0067$, Fig. 2a, Table S2). *Post-hoc* tests revealed that TS ($t = -7.9874$, $P < 0.0001$), SM ($t = -3.6784$, $P = 0.0012$), and the combined treatment TS+SM ($t = -7.2413$, $P < 0.0001$) all accelerated growth, compared to the control C (Table S3). The growth acceleration in TS, SM, and TS+SM was accompanied by a median advancement in embryonic stages of 3 to 9 hours, resulting in a switch from the segmentation to the pharyngula stage, compared to controls C and CM (see Fig. 2b, Supplementary Information, Fig. S1a-b, Tables S4-S5). Treatments had no obvious effect on mortality.

The control medium containing regularly excreted metabolites helped us to exclude oxygen saturation and pH as confounding effects. These levels were also independently measured at the beginning of treatments and in all cases fell within zebrafish natural tolerance ranges, with pH and oxygen levels ranging between [6.54-7.84] and [6.5-10.6 mg/L], respectively (Strecker et al., 2011; Zahangir et al., 2015). We therefore assessed whether stress medium, that is, the conditioned medium of embryos exposed to thermal stress, evoked different effects compared to control medium. Embryonic growth in CM was compared to growth in C and SM. Embryos in CM grew slightly slower than control embryos ($t = 2.3472$, $P = 0.0418$) and much slower than embryos in SM ($t = -6.7215$, $P < 0.0001$, Fig. 2a, Table S3).

Next, we investigated the behavioural startle-like response to light (Fig. 2c). Burst activity percentages were significantly lowered by both predictors stress medium ($H = 9.3222$, $P = 0.0023$) and thermal stress ($H = 17.008$, $P < 0.0001$), whereas the interaction term was not significant ($H = 1.8193$, $P = 0.1774$, Table S6). *Post-hoc* comparisons showed that embryos treated with SM ($W = 1,387$, $P = 0.0018$), TS ($W = 3,548$, $P = 0.0003$), and TS+SM ($W = 2,455$, $P < 0.0001$) all displayed lower burst percentages compared to control C. Embryos exposed to CM showed even stronger decline in burst activity percentages compared to C ($W = 3,287$, $P < 0.0001$) and SM ($W = 1,745$, $P < 0.0001$, Table S7).

Considering the aforementioned thermal stress-induced growth acceleration, stage-dependent effects of behaviour were investigated in control embryos incubated for 24, 31, 37, or 46 hrs. We observed that burst activity percentages significantly decreased with development in pre-hatching control embryos raised for 31, 37, and 46 hrs compared to those incubated for 24 hrs ($P < 0.0001$, Fig. S2c, Table S8). However, neither final stages ($P = 0.1627$) nor growth index ($P = 0.5027$) were correlated with burst activity percentages across treated embryos (Table S7). Moreover, pairwise tests showed that embryos under

development accelerated by TS, SM, and TS+SM treatments were significantly more active than control embryos reaching the same median stage of prim-6 in C31 ($P < 0.0001$, Fig. 2c, Table S9).

3.2. Effects of thermal stress and its propagation on stress response molecular biomarkers

First, we confirmed that HSP70 protein levels were increased in thermally-treated embryos, confirming that our treatment indeed caused heat stress (Supplementary Information, Fig. S2, Table S10). Secondly, the whole-embryo expression of three stress-inducible candidate genes (IL-1β, SOD1 and SQOR) was analysed (Fig. 3, Tables 1 and S11). Heat treatments were not a significant predictor variable for the expression of either IL-1β, SOD1, or SQOR. Pairwise comparison showed that SOD1 expression was slightly lowered in the TS treatment compared to the fresh medium control C (with borderline significance, $t = 2.76$, $P = 0.045$). Contrary to thermal stress treatments, stress medium SM did not increase HSP70 levels (Supplementary Information, Fig. S2, Table S10) but significantly reduced IL-1β expression (very large effect size, $t = 2.28$, $P = 0.038$) and increased SQOR expression (huge effect size, $t = -3.54$, $P = 0.003$) compared to fresh medium control C.

We then asked whether there are differences specifically between SM and CM. There were greater differences in the expression of IL-1β, SOD1 and SQOR between CM and treated embryos, than between C and treated embryos (Fig. 3). Pairwise tests between CM and SM conditions revealed significant differences in SOD1 (very large effect size, $t = -2.64$, $P = 0.034$), IL-1β (very large effect size, $t = 2.62$, $P = 0.034$) and there was also a trend for difference in SQOR expression ($P = 0.08$; Supplementary Table S11), indicating that SM and CM are fundamentally distinct. Taken together, our gene expression results suggest that incubation in the stress medium of previously heat-stressed embryos leads to altered antioxidant and immune responses in receiver embryos. On the other hand, neither thermal stress nor stress medium advanced the onset of the cortisol stress response in zebrafish embryos (Fig S3, Table S12).

4. Discussion

4.1. Fluctuating high temperatures induce a stress response in zebrafish embryos

The overarching aim of this work was to investigate whether zebrafish embryos can propagate aspects of their response to fluctuating heat stress to naive receiver embryos through conditioned media. First, we investigated the effects of fluctuating thermal stress on zebrafish embryos. Our results showed that embryos were indeed heat-stressed by our treatment, through higher HSP70 protein expression (Hallare et al., 2005). Heat stressed embryos grew faster than control embryos, which is consistent with previous reports (Long et al., 2012; Schnurr et al., 2014). This may in turn favour premature hatching of smaller larvae (Cingi et al., 2010; Schmidt and Starck, 2010). Stressed embryos were less active than control embryos incubated for 24 hrs, but were hyperactive compared to controls developed to the same stage of prim-6. Our results support previous observations that heat stress triggers higher behavioural activity in zebrafish early stages (Gau et al., 2013; Yokogawa et al., 2014) only compared to the aged-up control and the control medium, but not the fresh medium control. Such behavioural alterations may be explained by (i) energy trade-offs between behaviour, growth, and the metabolic costs of stress response (Barton and Iwama, 1991; Simčič et al., 2015), as well as (ii) temperature-dependent molecular changes in gene expression, epigenetic gene regulation, or post-translational modification related to behaviour, potentially involving circadian clock and neurodevelopmental genes (Colson et al., 2019; López-Olmeda and Sánchez-Vázquez, 2011).

At the gene expression level, we found that IL-1β and SQOR remained unchanged in thermally-stressed embryos. This contrasts with previous findings of SQOR upregulation in response to thermal and hypoxic stresses (Guo et al., 2016; Long et al., 2015, 2012; Xia et al., 2018) and of heat-induced increased IL-1β levels in adult black rockfish (*Sebastes schlegelii*; Lyu et al., 2018) and zebrafish embryos (Icoglu Aksakal and Ciltas, 2018). Higher temperature stress usually triggers an upregulation of SOD1 (Liu et al., 2018; Mahanty et al., 2016). In contrast, fluctuating thermal stress in this study reduced SOD1 expression only slightly compared to the control. These deviating results may result from measuring SOD1 expression not immediately after the cessation of thermal fluctuations. These inverted gene expression patterns under fluctuating as compared to constant thermal stress, might be related to energetic depletion as a result of the

thermal cycles (Alfonso et al., 2020; Schaefer and Ryan, 2006). Repetition of heat stress also attenuated the response of heat shock proteins at the mRNA level in lake whitefish (*Coregonus clupeaformis*) embryos (Sessions et al., 2021; Whitehouse et al., 2017). However, HSP70 protein levels remained upregulated by heat stress in our experiment. As gene expression varies with ontogeny during zebrafish embryogenesis (Mathavan et al., 2005), we could also have expected to find changes in gene expression in thermal treatments aligned to the acceleration of ontogeny, but we did not observe this. Collectively, our results confirm that fluctuating thermal stress induced a stress response in zebrafish embryos at the gene (marginally in SOD1 when compared between TS and C treatments), protein (HSP70), and phenotypic (growth, behaviour) levels. As cortisol is a biomarker for heat stress (Sadoul and Geffroy, 2019), one might expect that a stress-induced accelerated growth may also trigger an earlier onset of the cortisol stress response. However, cortisol levels remained unchanged in zebrafish embryos exposed to all treatments compared to control (Supplementary Information, Fig. S3, Table S12) which is concordant with previous literature reporting a cortisol stress response no earlier than 2-4 dpf in zebrafish (Alderman and Bernier, 2009; Alsop and Vijayan, 2009; Eto et al., 2014).

4.2. Thermal stress induces a positive stress feedback loop in naive receiver embryos

It is well-known that animals can chemically communicate a state of distress to others, although predation stress has traditionally received the most attention (Barcellos et al., 2014; Jordão and Volpato, 2000). Here, we found that fluctuating thermal stress negatively affects naive conspecifics, with a similar directionality of effects than the thermal stress itself. If this is the case, this could be described as a positive feedback loop (Fig. 1a). A feedback loop is defined as the outcome of a process which influences a future process within a system. Negative feedback loops are used to maintain homeostasis, while positive feedback loops amplify the outcome of the initial reaction.

First, embryos in our experiment grew faster when subjected to stress medium obtained from previously heat-stressed embryos, reaching similar stages to those of heat-stressed embryos. Such “catch-up” synchronous growth has been shown in egg-clustered embryos of several species and indicates the presence of embryo-embryo communication. Our results unexpectedly support the idea through the consistent differences observed between the control that only contained fresh E3 medium, and the control presumably containing regularly excreted metabolites. Usually this communication serves to maximise energy costs against potential threats and to potentially facilitate group emergence (Aubret et al., 2016; Colbert et al., 2010; McGlashan et al., 2012).

Second, stress medium triggered behavioural hyperactivity compared to control embryos reaching an equivalent stage (prim-6) and the control medium containing regular metabolites, but not the fresh medium control. These results are in agreement with higher activities in rainbow trout embryos facing alarm cues (Poisson et al., 2017) but depart from lower behaviour activities in 24 hpf zebrafish embryos exposed to conspecific alarm cues (Lucon-Xiccato et al., 2020), which suggest that the response depends on the type of the cue and the tested model. Behavioural alteration was also observed in adult zebrafish in response to low pH and fasting stress-induced metabolites (Abreu et al., 2016), and adult marine invertebrates experiencing metabolites from low pH-stressed conspecifics and heterospecifics (Feugere et al., 2021).

Third, stress medium conditioned by thermally-stressed embryos induced changes in gene expression patterns in naive conspecific receiver embryos. IL-1β was significantly downregulated in stress medium treatments. The stress medium-mediated inhibition of IL-1β in our experiment suggests that one of its inhibitor stress hormones, such as adrenaline and the adrenocorticotropic hormone (Castillo et al., 2009; Castro et al., 2011), or HSF1 (Cahill et al., 1996) - but not cortisol which remained unchanged here - could be upregulated by stress medium. Intriguingly, the expression of immune genes may be associated with behavioural changes in zebrafish, since highly responsive fish also have higher IL-1β expression (Kirsten et al., 2018). This may indicate a functional link between the concomitant decreases of IL-1β and lower activity of stress medium-treated embryos compared to embryos incubated in fresh medium for 24 hours.

On the other hand, SQOR expression was significantly upregulated by stress medium. SQOR emerged as a novel candidate marker from recent transcriptomics studies of thermal and oxidative stress (Guo et al., 2016; Long et al., 2015, 2012; Wollenberg Valero et al., 2021, in press; Xia et al., 2018). SQOR is involved

in the metabolism of hydrogen sulfide (H_2S), concentrations of which are toxic at supraphysiological levels, by regulating its neuromodulatory and biological roles (Augustyn et al., 2017; Chao et al., 2012; Horsman and Miller, 2016; Jackson et al., 2012; Rose et al., 2017). Interestingly, SQOR and H_2S may be involved in the response to oxidative stress through increasing glutathione levels (Kimura et al., 2010; Yonezawa et al., 2007) and by mediating the antioxidant effects of CoQ10 (Kleiner et al., 2018). There is evidence that SQOR maintains ATP production (Quinzii et al., 2017) and has been proposed as a growth-related candidate gene (Zhuang et al., 2020). Kleiner and colleagues (2018) found that an increase of SQOR may prevent oxidative stress by facilitating the antioxidant effects of CoQ10. Reversely, a downregulation of SQOR may reflect deficiency of its coenzyme CoQ10, which in turn alters the sulfide metabolism leading to accumulated H_2S levels and depletion of glutathione, that may cause oxidative damages (Luna-Sánchez et al., 2017; Quinzii et al., 2017; Ziosi et al., 2017). Therefore, the upregulation of SQOR under stress medium treatment could have multiple functions, from metabolising toxic levels of H_2S to restoring both ATP and GSH levels in response to stress communication from a conspecific. Altogether, our results may indicate impaired immune and antioxidant responses in embryos exposed to propagated thermal stress. Nevertheless, the molecular patterns, both at the gene (SOD, SQOR, IL-1β) and protein (HSP70, Fig. S2) levels suggest that the molecular pathways of action may differ between heat-stressed versus stress medium-conditioned embryos. Therefore, further work is needed to better understand the molecular pathways activated by the stress medium in comparison with thermal stress. Whilst a limitation of our experiment was that it only used a limited number of replicate pools of 60 embryos each to statistically compare the transcript levels, it should be emphasized that pooled sampling allows for confidence in averaged gene expression measurements (Schlötterer et al., 2014; Takele Assefa et al., 2020).

4.3. Stress medium and control medium do not induce similar feedback mechanisms

Embryos exposed to conditioned medium (by control embryos) developed slower than embryos exposed to fresh E3 medium only. These effects on development were mirrored by the expression patterns of the three investigated genes, which differed between control medium and experimental stress treatments. The behavioural response to stress medium was also different to that induced by the control medium, corroborating previous studies where metabolites from undisturbed versus stressed donors induced different responses in several species (Bairos-Novak et al., 2017; Bett et al., 2016). However, activity was much higher in control embryos from fresh E3 medium than in embryos incubated in control medium. This finding may indicate that behavioural activity of zebrafish embryos is tightly controlled by the nature of their chemical environment (vs. relaxed in the absence of any cues), lending additional support to chemical communication as a parameter relevant to thermal changes.

Lastly, the similarity in development and behaviour in combined thermal stress plus stress medium treatments, together with a larger effect size, indicate that thermal stress and stress medium may individually saturate the phenotypic reaction norms, which cannot be further altered in the combined treatment. Conversely, our gene expression and HSP70 analysis revealed a difference in molecular responses between the two independent factors, thermal stress and stress medium. To better understand these contrasting synergistic vs. independent effects of thermal stress and stress medium, gene expression could be compared at global scale in future work.

4.4. Conclusion

To conclude, our study indicates that thermally-stressed zebrafish embryos induce a stress-like response in naive conspecifics that have not been exposed to thermal stress, which is measurable from molecular to phenotypic levels. This phenomenon warrants designation as a positive feedback loop. If such positive feedback loops also exist in response to naturally occurring thermal fluctuations, this may have implications in the context of climate change or in high-density settings such as aquaculture. Further investigations should focus on the identification of stress metabolites potentially responsible for this stress communication, their molecular consequences at an individual level, as well as longer-term consequences for populations and ecosystems.

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Conflicts of Interest
The authors declare no conflicts of interest.

Ethics approval
All experiments were approved by the University of Hull Ethics committee under the approval U144b.

Consent to participate
All authors consented to participation.

Consent for publication
All authors consented to publication.

Availability of data and material (data transparency)
All data will be made publicly available upon manuscript acceptance.

Code availability (software application or custom code)
The R script used for data analysis will be made publicly available upon manuscript acceptance.

Author contribution
KWV conceived the study. KWV and PBA designed the experiments. LF, QRB, and VS collected the data and contributed to the statistical analysis. LF wrote the manuscript draft with PBA and KWV. All authors contributed to the final manuscript.

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Table

Table 1. Effect of thermal stress and stress medium on gene expression of pre-hatching zebrafish embryos. Effects of model terms (thermal stress and stress medium) on the expression of IL-1β, SOD1 and SQOR obtained via moderated t-tests (t-statistic) with *lmfit* and *eBayes* in the *limma* R package. B describes the log-odds of gene expression. Effect size is computed as Cohen's |d| and interpreted according to thresholds given in Sawilowsky (Sawilowsky 2009). Effect sizes of significant p-values ($P \leq 0.05$) are shown in bold.

Model terms	t	B	P	|d|	Effect size
IL-1β					
Thermal Stress	1.03	-4.70	0.310	0.49	medium
Stress Medium	2.28	-3.95	0.038	**1.20**	**very large**
SOD1					
Thermal Stress	1.65	-4.27	0.108	1.08	large
Stress Medium	0.32	-6.16	0.757	0.17	small
SQOR					
Thermal Stress	-0.17	-4.99	0.869	0.11	small
Stress Medium	-3.54	-1.67	0.003	**2.43**	**huge**

Figures

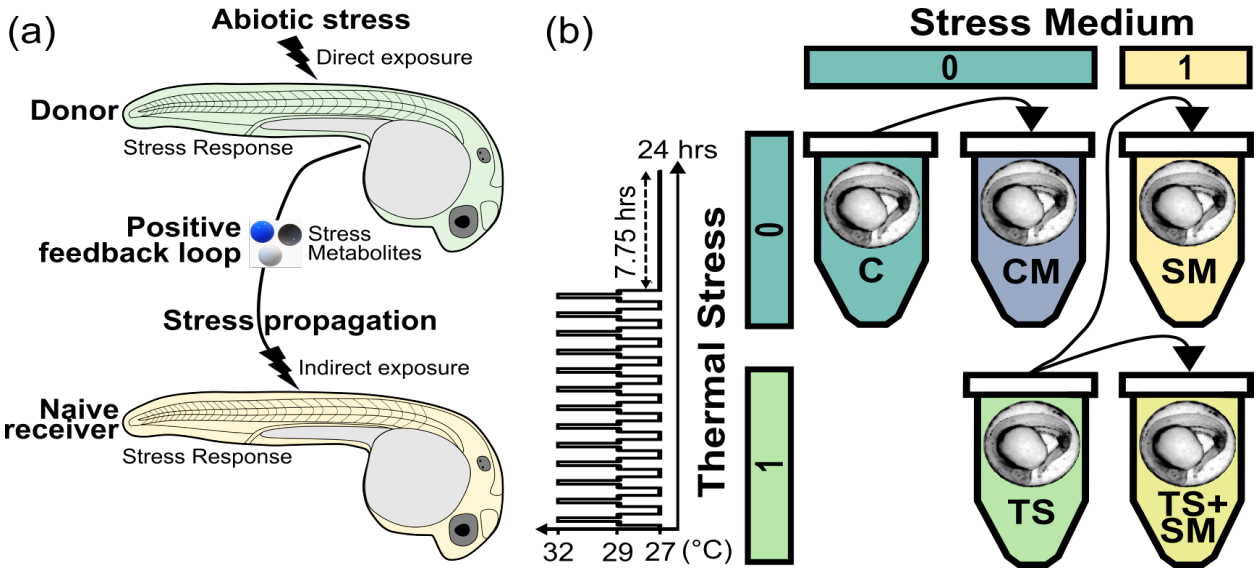

Figure 1. Overview of concept and experimental design. We tested the hypothesis that abiotic stress triggers the release of stress metabolites following a heat stress response, and that these stress metabolites signal a threat to naive receivers, in which they initiate an indirect stress response. Stress is propagated from directly exposed individuals towards naive conspecifics, thereby constituting a positive feedback loop. (b) In a two-way factorial design (represented by 0 and 1 for factor levels, respectively), zebrafish embryos (*Danio rerio*) were exposed to either fluctuating thermal stress (inset diagram) followed by a recovery period (dashed arrow line) or constant control temperature (27°C), in combination with fresh E3 medium or stress medium containing putative stress metabolites produced by one previously thermally-stressed embryo. An additional treatment was incubated at 27°C in a control medium containing metabolites from an embryo previously exposed to control conditions. Plain black arrows indicate medium transfer in which a new embryo was incubated. CM: control medium, C: control, SM: stress medium, TS: thermal stress, TS+SM: thermal stress + stress medium.

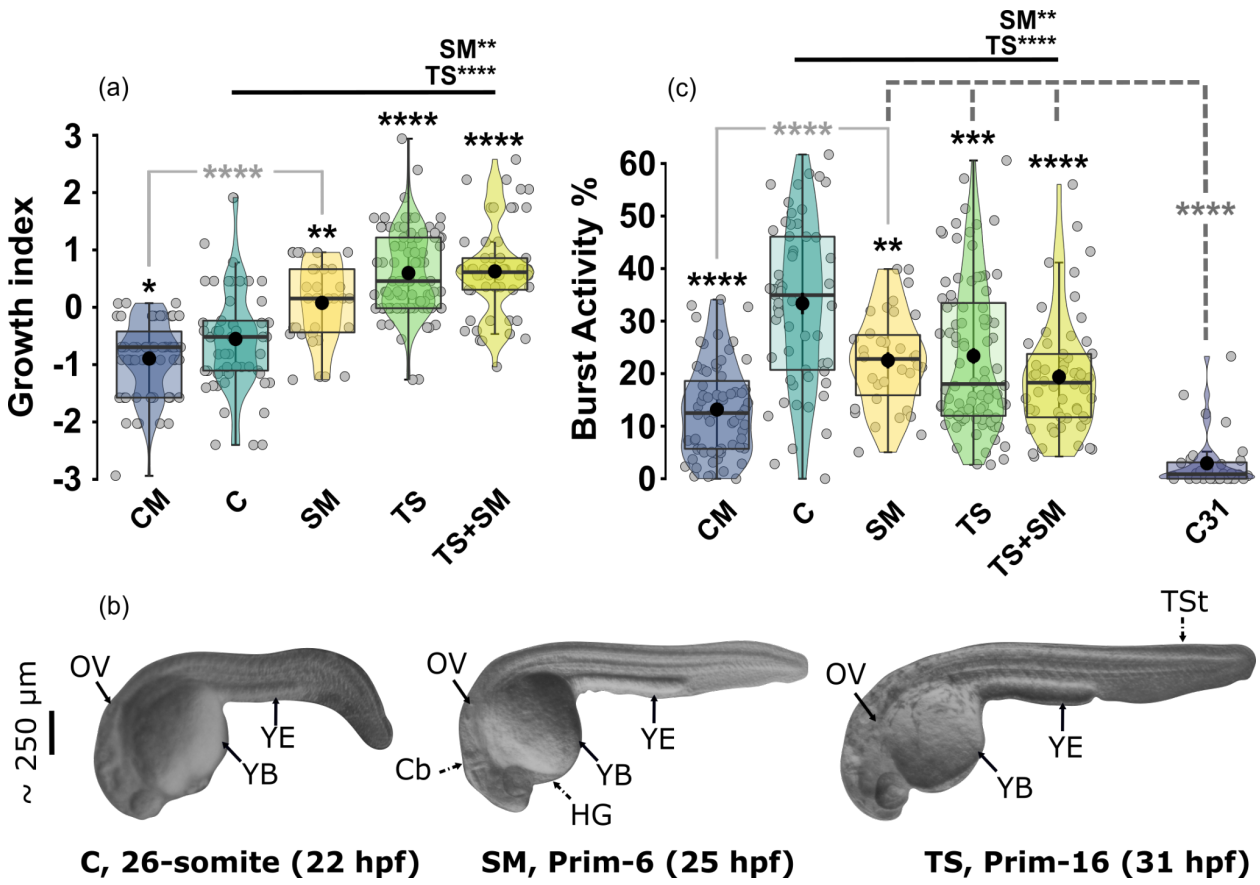

Figure 2. Thermal stress and stress medium accelerate the growth index (a) of pre-hatching zebrafish embryos, which are older in stress treatments (b), and alter the burst activity (c). CM: control medium (n = 67), C: control (n = 57), SM: stress medium (n = 34), TS: thermal stress (n = 90), TS+SM: thermal stress + stress medium (n = 56), C31: control after 31 hrs of incubation (reaching prim-6 stage). Boxes represent median, 25%-75% quartiles, and whiskers are minimum and maximum values within 1.5 IQR (interquartile range). Grey dots represent individual data points and black dots represent mean values. Effects of predictors (thermal stress and stress medium) were tested with two-factorial tests. Significant predictors are indicated in bold above horizontal black lines with *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. Significance of pairwise post−hoc tests is likewise indicated in asterisks, for treatments against C (bold black), between SM and CM (light grey), or between over-developing treatments (SM, TS, TS+SM) and C31 (dashed bar with dark grey asterisk). Fig. 2b: Representative images showing advanced developmental stages of stressed embryos at 1 dpf in stress medium (middle), and thermal stress (right), compared to control (left). Staging based on Kimmel et al (1995) using the otic vesicle (OV) length, the yolk extension (YE) to yolk ball (YB) ratio, and the presence of visible cerebellum (Cb, at prim-6), hatching gland (HG, at prim-6) or the straightening of the tail (TSt, at prim-16).

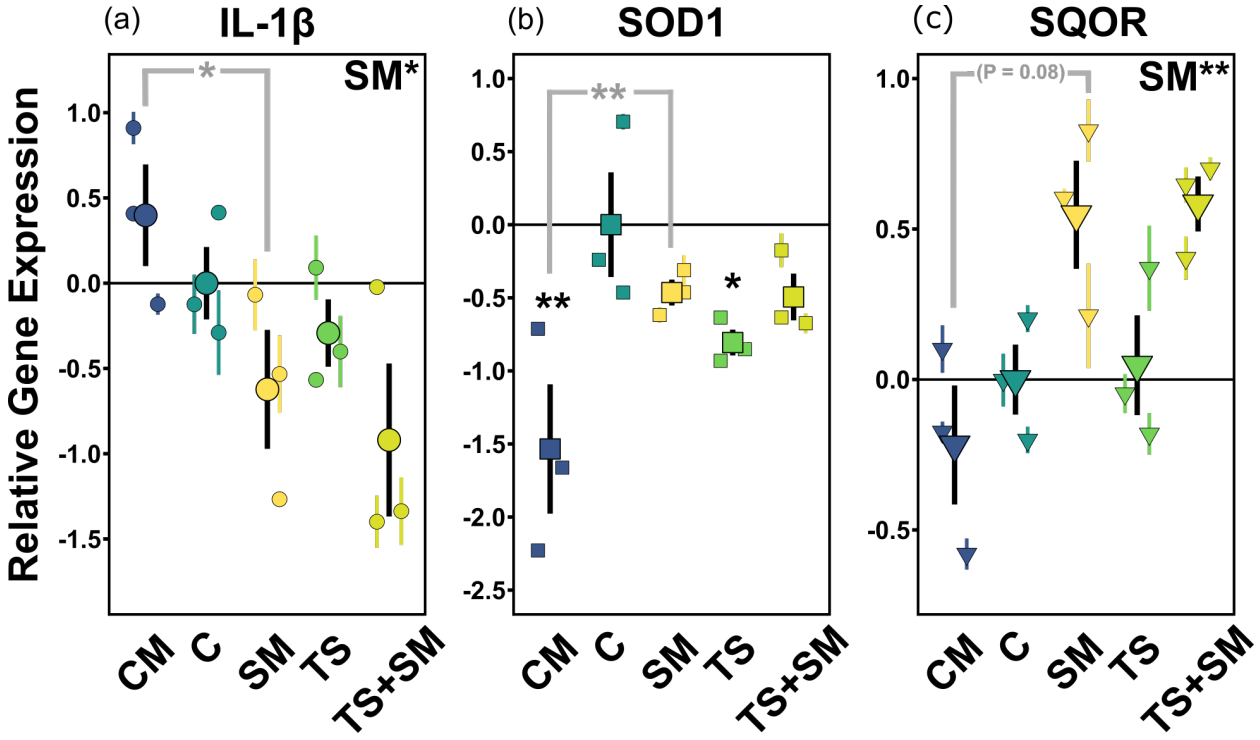

Figure 3. Stress medium alters stress-related gene expression of zebrafish embryos. (a) IL-1β (interleukin-1β), involved in immune response. (b) SOD1 (superoxide dismutase 1), involved in antioxidant response. (c) SQOR (sulfide:quinone oxidoreductase), involved in metabolism and antioxidant response. Jittered symbols represent mean $Log_2\ 2^{-\Delta\Delta CT} \pm$ SE (coloured bars) values of each biological replicate. Black symbols represent mean $Log_2\ 2^{-\Delta\Delta CT} \pm$ SE (black bars) values of each biological treatment. Expression is relative to control C (mean values as black horizontal lines). CM: control medium, C: control, SM: stress medium, TS: thermal stress, TS+SM: thermal stress + stress medium. Annotations on top right corners represent the factorial effects of thermal stress (TS) and stress medium (SM) on C, SM, TS, TS+SM. Black asterisks above each mean value only show the pairwise comparisons against the control C. Comparisons between CM and SM are represented in grey by asterisks and dashed horizontal lines. Statistics were computed using moderated t-tests (Limma, Bioconductor, R) with Bonferroni adjustment with *: $P \leq 0.05$, **: $P \leq 0.01$. Sample size: $n = 3$ biological replicate pools for each treatment each representing the average gene expression of 60 embryos per replicate.