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1 # Thermal stress induces positive phenotypic and molecular feedback loops in
2 # zebrafish embryos

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14 **Abstract**

15 Aquatic organisms must cope with both rising and rapidly changing temperatures. These environmental changes
16 can affect numerous traits, from molecular to ecological scales. Biotic stressors can induce the release of chemical
17 cues which trigger behavioural responses in other individuals. In this study, we infer whether abiotic stressors,
18 such as fluctuating temperature, may similarly propagate stress responses between individuals in fish not directly
19 exposed to the stressor. To test this hypothesis, zebrafish (*Danio rerio*) embryos were exposed for 24 hours to
20 fluctuating thermal stress, to medium in which another embryo was thermally stressed before ("stress medium"),
21 and to a combination of these. Growth, behaviour, and expression of a panel of genes were used to characterise
22 the thermal stress response and its propagation between embryos. Both high temperatures and stress medium
23 significantly accelerated development and altered embryonic behaviour. Thermal stress significantly decreased
24 the expression of the antioxidant gene SOD1, eight hours after the end of exposure. Of note, we found that the
25 expression of sulfide:quinone oxidoreductase (SQOR), likewise a part of the antioxidant metabolism relevant in
26 vertebrate stress response, and of interleukin-1β (IL-1β), involved in the immune response, were significantly
27 altered by stress medium. This study illustrates the existence of positive thermal stress feedback loops in zebrafish
28 embryos that induce stress in conspecifics. This evidence that thermal stress due to fluctuating, high temperatures
29 can be propagated may be relevant for species found in high densities, either in aquaculture or in the natural
30 environment, in a context of global change.

31

32 **Keywords:** behaviour, chemical communication, climate warming, gene expression, feedback loop, thermal
33 stress, zebrafish embryos

34

35 **Introduction**

36 Temperature is the abiotic 'ecological master factor' regulating the biology of ectotherms (Brett, 1971; López-
37 Olmeda & Sánchez-Vázquez, 2011). Ectotherms face thermal cycles that shape their biological rhythms by
38 modulating survival, growth, and by triggering irreversible changes on thermal tolerance ranges of adults (Colinet
39 et al., 2015; Kingsolver et al., 2015; Lim et al., 2017; López-Olmeda & Sánchez-Vázquez, 2011; Schaefer & Ryan,
40 2006). Those natural, regular thermal fluctuations experienced during early development are necessary to
41 maximise fitness-relevant traits and thermal tolerance (Hall & Warner, 2020b; Kingsolver et al., 2015; Lim et al.,
42 2017; Schaefer & Ryan, 2006). Besides higher average temperatures, global warming will be characterised by
43 both greater thermal variability and more extreme thermal events of longer duration and magnitude (Pörtner et al.,
44 2019; Vasseur et al., 2014). These altered thermal rhythms may pose higher risks to species than simply higher
45 mean temperatures, due to the asymmetry of the thermal fitness curve (Colinet et al., 2015; Saxon et al., 2018;
46 Vasseur et al., 2014). Here, we exposed zebrafish embryos to several peaks of higher temperatures that consisted
47 in thirteen exposures to +5°C within 24 hours. Although this thermal regime departs from natural, environmental

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48 relevant extreme heatwaves, it serves to model the effects of repeated thermal stress upon the stress response of
49 zebrafish embryos.
50 Fish as ectothermic vertebrates are susceptible to changes in the thermal environment, particularly to higher
51 temperatures close to their upper thermal limits (Araújo et al., 2013; I. J. Morgan et al., 2001; Paaijmans et al.,
52 2013). Early developmental stages have narrower thermal ranges than adults (Skjærven et al., 2014). Temperature
53 regimes during development have irreversible effects as they modulate subsequent stages, making early fish
54 embryos vulnerable or "bottleneck" stages in the context of climate change (Pörtner & Peck, 2010; Scott &
55 Johnston, 2012; Villamizar et al., 2012). Examples for persistent effects of temperature changes during
56 development may involve alterations of swimming performance and cardiac anatomy (Dimitriadi et al., 2018),
57 masculinisation (Ribas et al., 2017) and increased mortality (Hosseini et al., 2019), which can shape the future
58 trajectory of populations. Altogether, this illustrates a rising concern about the response of fish to global change,
59 particularly at early stages of development.
60 The zebrafish (*Danio rerio*) is now emerging as a model organism to study the effects of thermal stress, including
61 at the molecular level (Brown et al., 2015; Clark et al., 2011; Long et al., 2012; Luu et al., 2020). Adult zebrafish
62 are eurythermal, naturally tolerating a wide range of temperatures (16.5-38.6°C) with optimal temperature around
63 27-28.5°C. They may face natural thermal variations of ~5°C daily, and from 6 to 38°C seasonally (Engeszer et
64 al., 2007; López-Olmeda & Sánchez-Vázquez, 2011; Spence et al., 2008). However, early stages of zebrafish only
65 tolerate minimum temperatures of 22-23°C and maximum temperatures of around 32°C to develop normally (Pype
66 et al., 2015; Schirone & Gross, 1968; Schnurr et al., 2014). Warm-adapted species such as the zebrafish that are
67 living near their upper thermal limit may be among the 'losers' of climate change (Somero, 2010). Of note, the
68 thermal biology of zebrafish is conserved in laboratory populations, in spite of laboratory domestication, which
69 makes them an adequate model organism to investigate the effects of climate change in the laboratory (Brown et
70 al., 2015; R. Morgan et al., 2019).
71 Besides the above-mentioned effects on sex ratio, direct mortality and adult phenotype, responses to thermal stress
72 at the early stages of development involve altered behaviour, developmental rate, and altered gene expression
73 related to the physiological stress response. Behavioural thermoregulation is one major thermoregulatory process
74 in ectotherms (López-Olmeda & Sánchez-Vázquez, 2011). This is evident from a tightly controlled thermotaxis
75 response in response to heat in zebrafish larvae (Haesemeyer et al., 2015, 2018). Heat stressed zebrafish larvae
76 display more anxiety-like behaviours (Bai et al., 2016) and transient hyperactivity (Yokogawa et al., 2014). High
77 temperatures also accelerate the embryonic development in zebrafish embryos as shown by the 2.8-fold increase
78 in somitogenesis frequency across a 20-30°C range or the twice faster development at 33°C compared to 26°C
79 (Hallare et al., 2005; Long et al., 2012; Schröter et al., 2008). Repeated exposure to sublethal temperatures,
80 however, may depress development (Hall & Warner, 2020a). At the molecular level, heat stress leads to numerous
81 molecular effects from the accumulation of reactive oxygen species (ROS) (Madeira et al., 2016; Vinagre et al.,
82 2012) to changes in global gene expression patterns (Logan & Buckley, 2015; Long et al., 2012; Ribas et al., 2017)
83 along the hypothalamic-pituitary-interrenal (HPI) axis (Alsop & Vijayan, 2009). For example, Cu/Zn-superoxide

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84 dismutase I (SOD1) neutralises oxygen radicals to protect cells from oxidative stress (Cheng et al., 2018; Wang et
85 al., 2016), and is heat inducible in zebrafish embryos (Icoglu Aksakal & Ciltas, 2018). Another less well studied
86 enzyme, sulfide:quinone oxidoreductase (SQOR) is upregulated by heat stress in half-smooth tongue sole
87 (*Cynoglossus semilaevis*) (Guo et al., 2016) and by hypoxia in Nile tilapia (*Oreochromis niloticus*) (J. H. Xia et
88 al., 2018). SQOR expression is co-induced by both cold stress and hypoxia in zebrafish embryos (Long et al.,
89 2012, 2015). The innate immune system is challenged by both high temperature and thermal fluctuations in teleost
90 early stages (Mariana & Badr, 2019; Zhang et al., 2018). For instance, interleukin-1β (IL-1β) is upregulated in
91 zebrafish raised at high temperature but experiencing an immune challenge at low temperature (Zhang et al., 2018)
92 and in zebrafish embryos exposed to high temperature (Icoglu Aksakal & Ciltas, 2018). This gene plays a central
93 role in the stress response, having neuromodulatory and behavioural functions (Goshen & Yirmiya, 2009; Metz et
94 al., 2006; Vitkovic et al., 2000).
95 Largely overlooked to date, however, is the question of whether the response to thermal stress can be transmitted
96 to other individuals. Fish use chemical communication to alert others of a threat using so-called 'alarm cues'
97 (released after skin damage) or 'disturbance cues' (released without injury following a biotic stressor) (Jordão &
98 Volpato, 2000). Exposure to conspecific predation-related disturbance cues induces stress-like responses in several
99 fish species (Barcellos et al., 2011, 2014; Bett et al., 2016; Ferrari et al., 2008; Jordão & Volpato, 2000; Toa et al.,
100 2004), including zebrafish (Barcellos et al., 2014). Importantly, even fish early stages are capable of such chemical
101 communication as they respond to alarm cues (Atherton & McCormick, 2015, 2017; Oulton et al., 2013; Poisson
102 et al., 2017). Despite the wealth of information on biotic stress-induced stress propagation, this phenomenon is
103 only known from a few abiotic stressors, such as low pH, acute fasting or handling (Abreu et al., 2016; Feugere et
104 al., in review), but in response to heat is only known from crayfish (Hazlett, 1985). Stress induces the release of
105 metabolites into the environment, including hormones of the HPI axis (Barcellos et al., 2014; McGlashan et al.,
106 2012), CO_2 (McGlashan et al., 2012), respiratory byproducts, catecholamines, or nitrogenous metabolic products
107 such as urea (Bairos-Novak et al., 2017; Giaquinto & Hoffmann, 2012; Henderson et al., 2017; Hubbard et al.,
108 2003), but less is known about their effects on communication. Distinct from alarm and disturbance cues, we
109 introduced the term 'stress metabolite' referring to such cues induced without injury as a byproduct of the response
110 to abiotic stress (Feugere et al., in review).
111 In this contribution we infer whether thermal stress can be propagated to naive receivers. To test this hypothesis,
112 zebrafish embryos were exposed to independent and combined treatments of thermal stress and medium putatively
113 containing stress metabolites induced by this thermal stress. Zebrafish embryo behaviour, growth rate, and
114 expression of genes involved in the immune response (IL-1β) and antioxidant pathways (SOD1, SQOR) were
115 investigated. We expected that (i) fluctuating high temperatures, similar to constant higher temperatures, trigger
116 developmental, behavioural and molecular stress responses in zebrafish embryos. Second (ii), we hypothesised
117 that these responses could induce a positive feedback loop in naive receiver embryos, which are however (iii) not
118 elicited by non-stress metabolites. It should be emphasised that our work primarily aimed to investigate the

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119 existence of stress propagation induced by repeated thermal stress, in a context of growing concern for a future
120 more stressful environment, rather than study the effects of realistic heatwaves.
121

122 Materials and Methods

123 Experimental design

124 For detailed zebrafish husbandry and breeding methods, see Supplementary Information. Just before the beginning
125 of experimental treatments, several zebrafish embryos per clutch were photographed to estimate the median initial
126 stage. Exposure began around the blastula stage (median stage: 2.75 hpf, at 512-cells). Fertilised healthy embryos
127 (with chorion) were selected and individually placed into 0.2 mL 8-strip PCR tubes prefilled with 200 µL of 1X
128 E3 medium.
129 In a two-factorial design, embryos were exposed to different combinations of the two factors, temperature stress
130 and temperature-induced stress medium. For this purpose, embryos were exposed to either thermal stress or control
131 temperature protocols within a thermocycler, in either fresh medium or medium where embryos had previously
132 been exposed to thermal stress and containing putative stress metabolites. All experimental treatments are detailed
133 in Figure 1 and Table S1. The thermal stress protocol spanned 16.25 hrs, divided into thirteen 75-min series of
134 temperature fluctuations between 27, 29, 32, 29, and 27°C, with each temperature step being maintained for 15
135 min. Thermal stress mimicked +5°C temperature peaks over zebrafish optimal temperature (a total of n = 13 peaks)
136 reaching the sublethal temperature of 32°C (Scott & Johnston, 2012). A recovery time of 7.75 hrs at 27°C followed
137 the fluctuating temperatures period to reach a total incubation time of 24 hrs. The control thermal protocol was a
138 steady 27°C for 24 hrs. E3 media following control or thermal stress conditions were reused for 'control medium'
139 and 'stress medium' treatments, respectively. In total this yielded five treatments: control (C, control protocol with
140 fresh medium), control medium (CM, control protocol with reused medium from C), thermal stress (TS, thermal
141 stress protocol with fresh medium), stress medium (SM, control protocol with reused medium from TS), and
142 thermal stress + stress medium (TS+SM, thermal stress protocol with reused medium from TS). Used media were
143 immediately re-used for treatments containing putative stress or control metabolites, respectively. As development
144 in zebrafish accelerates with higher temperature (Kimmel et al., 1995) but decelerates with darkness (Bucking et
145 al., 2013; Villamizar et al., 2014), additional control embryos were monitored after longer incubation times: 31
146 hrs (C31), 37 hrs (C37), and 46 hrs (C46). These times were adjusted for darkness-raised embryos to reach the
147 stages of prim-6 (25 hpf), prim-16 (31 hpf), and late pharyngula (35 - 42 hpf), respectively. Initial, final, and total
148 exposure times were used to standardise each procedure. After incubation, transparent embryos were deemed alive
149 and kept for subsequent steps. Before and after each exposure, embryo media were sampled to measure pH and
150 O_2 saturation levels that could impact embryos in the used media. Oxygen levels were averaged from 1-2 min
151 measurements using a glass vial equipped with an oxygen sensor spot (OXSP5, sensor code: SC7-538-193,
152 Pyroscience GmbH) and connected to the FireSting O_2 Fiber Optic Oxygen Meter (FSO2-4, Pyroscience GmbH)
153 and Oxygen Logger software (Pyroscience GmbH). Constant medium pHs were verified using the Fisherbrand™
154 accumet™ AB150 pH Benchtop Meters.

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155

156 **Analysis of phenotypic data**

157 Phenotypic analyses were conducted for all eight treatments. Embryos were placed in a watch glass vial and
158 videoed by small batches using the camera setup (see Supplementary Information) over 15-30 seconds. Embryos
159 were placed under light (similar intensity across measurements) to elicit a startle-like response after exposure in
160 darkness. Behavioural data was analysed using Danioscope (Noldus). When possible, several videos were recorded
161 for each embryo clutch and behavioural measurements were averaged for each individual embryo. Analysis of
162 behavioural responses were conducted on the video-length standardised burst activity percentage (percentage of
163 the time – from the total measurement duration – the embryo was moving). Final embryonic stages were estimated
164 following Kimmel et al. (1995) from several photographs of embryos within their chorions using the criteria somite
165 number, yolk extension to yolk ball ratio, presence and morphology of otoliths, tail aspect, presence of lens
166 primordium, presence and position of the cerebellum relatively to the eyes, and pigmentation. The growth index
167 was calculated as in equation 1.

168 $$\frac{\Delta hpf}{hr} = \frac{(embryo\ final\ stage\ (hpf) - batch\ median\ initial\ stage\ (hpf))}{batch\ exposure\ time\ (hr)} \quad \text{(equation 1)}$$

169 Statistical analyses were conducted in RStudio (RStudio Team, 2020). Outliers among behavioural values were
170 excluded from statistical analyses using Tukey's method with a 1.5 interquartile range cut-off. First, the effects of
171 stress medium and thermal stress predictors across the C, TS, SM, and TS+SM treatments were evaluated. Shapiro-
172 Wilk and Levene's tests were used to evaluate normality and homogeneity of variances, respectively.
173 Normalisation methods were compared using the *BestNormalize* R package (Peterson & Cavanaugh, 2020). The
174 growth index was parameterised using an order normalising transformation (Shapiro-Wilk's P = 0.98, Levene's P
175 = 0.27) and analysed by a two-way ANOVA with thermal stress and stress medium as predictors, followed by
176 *post-hoc* pairwise Student's t-tests between control C and each experimental condition. The burst activity
177 percentage and final stage (in hpf) could not be normalized and were analysed for the effects of both predictors
178 using nonparametric Scheirer-Ray-Hare tests from the *rcompanion* R package (Mangiafico, 2018), followed by
179 pairwise *post-hoc* Wilcoxon-Mann-Whitney tests. The final embryonic developmental period was coded as
180 *segmentation* or *pharyngula* and analysed for the effects of thermal stress, stress medium as factors and initial
181 stages as covariate using a generalised logistic model followed by pairwise *post-hoc* comparisons using the
182 *emmeans* R package (Lenth, 2019).

183 Additional pairwise comparisons were performed of all response variables of CM against C and SM. To determine
184 whether behavioural effects of treatment were related to developmental acceleration, pairwise comparisons of
185 behaviour of control embryos (C) were compared to that of older control embryos (C31, C37, C46) using Kruskal-
186 Wallis tests. Fourth, burst activity percentages of embryos from stress medium and thermal stress treatments were
187 compared against that of control embryos incubated for longer times using pairwise Wilcoxon-Mann-Whitney
188 tests. Multiple comparisons were corrected using Bonferroni adjustments. Cohen's |d| were obtained from the
189 distribution used to compute the statistical analyses and calculated using the *effsize* r package (Torchiano, 2016)

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190 or according to Lenhard & Lenhard (2016), respectively. Effect size was qualified based on thresholds given in
191 Sawilowsky (2009): very small: $|d| > 0.01$, small: $|d| > 0.2$, medium: $|d| > 0.5$, large: $|d| > 0.8$, very large: $|d| > 1.20$,
192 huge: $|d| > 2.0$.
193

194 **Gene expression**

195 Gene expression analyses were conducted for CM, C, SM, TS, and TS+SM treatments (n = 3 pooled biological
196 replicates of 60 embryos per treatment). Embryos were snap-frozen at -80°C immediately after experimental
197 treatments. Total RNA was extracted using a High Pure RNA isolation kit (Sigma-Aldrich) following the
198 manufacturer's recommendations. cDNA was synthesized using Superscript II™ Reverse Transcriptase
199 (Invitrogen, Life Technologies Ltd.) with sample randomisation. TaqMan® Gene Expression Assays
200 (ThermoFisher Scientific) and 2X qPCR Bio Probe Hi-ROX (PCRBiosystems) were used to quantify the
201 expression of three genes of interest (SQOR, SOD1, and IL-1β) normalised to two reference genes (ef1-α, β-actin).
202 The effects of stress medium and thermal stress on the $Log_2\ 2^{-\Delta\Delta CT}$ (Log_2 fold-change) values were investigated
203 using the *eBayes* and *lmFit* functions within the *limma* package (Ritchie et al., 2015) within the *Bioconductor*
204 *v.3.11* (Ihaka & Gentleman, 1996) R environment. Next, pairwise *post-hoc* comparisons on C *versus* SM, TS, or
205 TS+SM, and CM *versus* C or CM were performed using pairwise moderated t-tests with Bonferroni adjustments.
206 Effect sizes (Cohen's $|d|$) were calculated as above. More details on the gene expression analysis are given as
207 Supplementary Information.
208

209 **Results**

210 **Phenotypic effects of thermal stress and its propagation**

211 First, the phenotypic effects of fluctuating thermal stress and of stress medium treatments were analysed.
212 Embryonic growth indices were significantly accelerated by stress medium (small effect size, F = 6.291, P =
213 0.0128), thermal stress (large effect size, F = 75.502, P < 0.0001), and their combination (very large effect size, F
214 = 7.498, P = 0.0067, Figure 2a, Table 1). *Post-hoc* tests revealed that TS (t = -7.9874, P < 0.0001), SM (t = -
215 3.6784, P = 0.0012), and the combined treatment TS+SM (t = -7.2413, P < 0.0001) all accelerated growth,
216 compared to the control C (Table S2). The growth acceleration was accompanied by a median advancement in
217 embryonic stages of 3 to 9 hours, resulting in a switch from the segmentation to the pharyngula stage, compared
218 to controls C and CM (see Supplementary Information, Figure S1a-b, Tables S3-S4). Treatments had no obvious
219 effect on mortality.
220 We reasoned that any observed effects of stress medium may be due to regularly excreted metabolites, which
221 accumulate towards the end of each treatment independently of thermal stress and would be up-concentrated in
222 SM and TS+SM treatments. This warranted the use of an additional control, the 'control medium' (CM), that is,
223 medium that had previously hosted control embryos. This control helped us exclude oxygen saturation and pH as
224 confounding effects, which were also independently measured at the beginning of treatments and in all cases fell
225 within zebrafish natural tolerance ranges (Strecker et al., 2011; Zahangir et al., 2015). We therefore assessed

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226 whether stress medium, that is, medium where embryos had been exposed to thermal stress, evoked different
227 effects compared to control medium. Embryonic growth in CM was compared to growth in C and SM. Embryos
228 in CM grew slightly slower than control embryos ($t = 2.3472$, $P = 0.0418$) and much slower than embryos in SM
229 ($t = -6.7215$, $P < 0.0001$, Figure 2a, Table S2).
230 Next, we investigated the behavioural startle-like response to light (Figure 2b). Burst activity percentages were
231 significantly lowered by both predictors stress medium ($H = 9.3222$, $P = 0.0023$) and thermal stress ($H = 17.008$,
232 $P < 0.0001$), whereas the interaction term was not significant ($H = 1.8193$, $P = 0.1774$, Table 2). *Post-hoc*
233 comparisons showed that embryos treated with SM ($W = 1{,}387$, $P = 0.0018$), TS ($W = 3{,}548$, $P = 0.0003$), and
234 TS+SM ($W = 2{,}455$, $P < 0.0001$) all displayed lower burst percentages compared to control C. Embryos exposed
235 to CM showed even stronger decline in burst activity percentages compared to C ($W = 3{,}287$, $P < 0.0001$) and SM
236 ($W = 1{,}745$, $P < 0.0001$, Table S5).
237 Considering the aforementioned thermal stress-induced growth acceleration, stage-dependent effects of behaviour
238 were investigated in control embryos incubated for 24, 31, 37, or 46 hrs. We observed that burst activity
239 percentages significantly decreased with development in pre-hatching control embryos raised for 31, 37, and 46
240 hrs compared to those incubated for 24 hrs ($P < 0.0001$, Figure S2c, Table S6). However, neither final stages ($P =$
241 0.1627) nor growth index ($P = 0.5027$) were correlated with burst activity percentages across treated embryos
242 (Table S5). Moreover, pairwise tests showed that stressed embryos under development accelerated by TS, SM,
243 and TS+SM treatments were significantly more active than control embryos reaching the same median stage of
244 prim-6 in C31 ($P < 0.0001$, Figure 2b, Table S7).
245
246 **Effects of thermal stress and its propagation on stress response-related gene expression**
247 The whole-embryo expression of three stress-inducible candidate genes (IL-1β, SOD1 and SQOR) was analysed
248 (Figure 3, Tables 3 and S8). Thermal stress was not a significant predictor for the expression of either IL-1β or
249 SQOR, but SOD1 expression was lower in the TS treatment compared to the control C (very large effect size but
250 marginal significance, $t = 2.76$, $P = 0.045$). In contrast, stress medium significantly reduced IL-1β expression (very
251 large effect size, $t = 2.28$, $P = 0.038$) and increased SQOR expression levels (huge effect size, $t = -3.54$, $P = 0.003$),
252 but had no effects on SOD1 expression.
253 Pairwise tests revealed that SOD1 gene expression in control medium (CM) was much lower compared to C (huge
254 effect size, $t = -3.78$, $P = 0.003$), but did not differ from C for the expression of SQOR and IL-1β. Second, pairwise
255 tests showed that stress medium treatment (SM) triggered different gene expression responses compared to CM in
256 all three studied markers. SM significantly reduced the expression of IL-1β (very large effect size, $t = 2.62$, $P =$
257 0.034) and increased that of SOD1 (very large effect size, $t = -2.64$, $P = 0.034$), whereas there was a only a non-
258 significant trend towards increased SQOR expression ($P = 0.08$). Relative to the control, the gene expressions of
259 IL1-β and SQOR in SM showed opposite patterns compared to CM. Taken together, our gene expression results
260 suggest that incubation in stress medium leads to reduced IL-1β expression and increased SQOR expression.
261

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262 ## Discussion

263 **Fluctuating high temperatures induce a stress response in zebrafish embryos**

264 Communication between conspecifics can potentiate the response of a group to stressors (Giacomini et al., 2015),
265 consequently the combined effects of abiotic stressors and any feedback loops they induce are of great importance
266 in the context of global change. For instance, by the end of the century not only temperature but also chemical
267 cues associated with a warming aquatic environment could be altered (Chivers et al., 2013; Lienart et al., 2016;
268 Roggatz et al., 2019), and warming is known to affect predator-prey communication via infochemicals (J. Xia et
269 al., 2020). The overarching aim of this work was to investigate whether zebrafish embryos can propagate aspects
270 of their response to fluctuating heat stress to naive receiver embryos through positive feedback loops.
271 First, we investigated the effects of fluctuating thermal stress on zebrafish embryos. Our results showed that heat
272 stressed embryos grew faster than control embryos, which is consistent with previous reports (Long et al., 2012;
273 Schnurr et al., 2014). This may in turn favour premature hatching of smaller larvae (Cingi et al., 2010; Schmidt &
274 Starck, 2010). Stressed embryos were less active than control embryos incubated for 24 hours but were hyperactive
275 compared to controls developed to the same stage of prim-6 without stress treatments. Our results support previous
276 observations that heat stress triggers higher behavioural activity in zebrafish early stages (Gau et al., 2013;
277 Yokogawa et al., 2014). Such behavioural alterations may be explained by (i) energy trade-offs between behaviour,
278 growth, and the metabolic costs of stress response (Barton & Iwama, 1991; Simčič et al., 2015), as well as (ii)
279 temperature-dependent molecular changes in gene expression, epigenetic gene regulation, or post-translational
280 modification related to behaviour, potentially involving circadian clock and neurodevelopmental genes (Colson et
281 al., 2019; López-Olmeda & Sánchez-Vázquez, 2011).
282 At the gene expression level, we found that IL-1β and SQOR remained unchanged in thermally-stressed embryos.
283 This contrasts with previous findings of SQOR upregulation in response to thermal and hypoxic stresses (Guo et
284 al., 2016; Long et al., 2012, 2015; J. H. Xia et al., 2018) and of heat-induced increased IL-1β levels in adult black
285 rockfish (*Sebastes schlegelii*) (Lyu et al., 2018) and zebrafish embryos (Icoglu Aksakal & Ciltas, 2018). Higher
286 temperature stress usually triggers an upregulation of SOD1 (Liu et al., 2018; Mahanty et al., 2016). In contrast,
287 fluctuating thermal stress in this study reduced SOD1 expression compared to the control, measured almost eight
288 hours after the cessation of thermal fluctuations. These inverted gene expression patterns under fluctuating, as
289 compared to constant thermal stress, might be related to energetic depletion as a result of the thermal cycles
290 (Schaefer & Ryan, 2006). Repetition of heat stress also downregulated heat shock proteins in lake whitefish
291 (*Coregonus clupeaformis*) embryos (Whitehouse et al., 2017). Nevertheless, it should be acknowledged that the
292 differences in developmental rate observed under fluctuating heat stress may superimpose confounding effects to
293 the effects of the heat stimulus, as gene expression varies with ontogeny during zebrafish embryogenesis
294 (Mathavan et al., 2005). Nonetheless, it is likely that fluctuating temperatures experienced through early
295 development of zebrafish embryos cause lasting developmental and behavioural changes, similar to those already
296 shown to occur in vertebrate ectotherms after static increased temperatures.
297

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298 **Thermal stress induces positive stress feedback loops in naive receiver embryos**

299 It is well-known that animals can chemically communicate a state of distress to others, although predation stress
300 has traditionally received the most attention (Barcellos et al., 2014; Jordão & Volpato, 2000). Here, we found that
301 fluctuating thermal stress negatively affects naive conspecifics, with a similar directionality of effects than the
302 thermal stress itself, which can be described as a positive feedback loop.

303 First, embryos in our experiment grew faster when subjected to stress medium obtained from previously heat-
304 stressed embryos, reaching similar stages to those of heat-stressed embryos. Such "catch-up" synchronous growth
305 has been shown in egg-clustered embryos of several species and indicates the presence of embryo-embryo
306 communication. Usually this communication serves to maximise energy costs against potential threats and to
307 potentially facilitate group emergence (Aubret et al., 2016; Colbert et al., 2010; McGlashan et al., 2012).

308 Second, stress medium triggered behavioural hyperactivity compared to control embryos reaching an equivalent
309 stage (prim-6). These results are in agreement with higher activities in rainbow trout embryos facing alarm cues
310 (Poisson et al., 2017) but depart from lower behaviour activities in 24 hpf zebrafish embryos exposed to
311 conspecific alarm cues (Lucon-Xiccato et al., 2020), which suggest that the response depends on the type of the
312 cue and the tested model. In our experiment, these behavioural responses were measured, for the first time, as a
313 response to temperature stress-elicited cues. Behavioural alteration was also observed in adult zebrafish in
314 response to low pH and fasting stress-induced metabolites (Abreu et al., 2016), and adult marine invertebrates
315 experiencing metabolites from low pH-stressed conspecifics and heterospecifics (Feugere et al, in review).

316 Third, stress medium originating from thermally-stressed embryos induced changes in gene expression patterns in
317 naive conspecific receiver embryos. IL-1β was significantly downregulated in stress medium treatments. The stress
318 medium-mediated inhibition of IL-1β in our experiment suggests that one of its inhibitor stress hormones, such as
319 cortisol, adrenaline and the adrenocorticotropic hormone (Castillo et al., 2009; Castro et al., 2011), or HSF1 (Cahill
320 et al., 1996) could be upregulated by stress media. Intriguingly, the expression of immune genes may be associated
321 with behavioural changes in zebrafish, since highly responsive fish also have higher IL-1β expression (Kirsten et
322 al., 2018). This indicates a functional link between the concomitant decreases of IL-1β and lower activity of stress
323 medium-treated embryos compared to 24 hrs-incubated medium control embryos.

324 On the other hand, SQOR expression was significantly upregulated by stress medium. SQOR has been little studied
325 in an environmental stress response context thus far, but emerged as a novel candidate marker from recent
326 transcriptomics studies of thermal and oxidative stress (Guo et al., 2016; Long et al., 2012, 2015; Wollenberg
327 Valero et al., 2019; J. H. Xia et al., 2018). SQOR is involved in the metabolism of hydrogen sulfide (H_2S),
328 concentrations of which are toxic at supraphysiological levels, by tuning its neuromodulatory and biological roles
329 (Augustyn et al., 2017; Chao et al., 2012; Horsman & Miller, 2016; Jackson et al., 2012; Rose et al., 2017).
330 Interestingly, SQOR and H_2S may be involved in the response to oxidative stress through increasing glutathione
331 levels (Kimura et al., 2010; Yonezawa et al., 2007) and by mediating the antioxidant effects of CoQ10 (Kleiner et
332 al., 2018). There is evidence that SQOR maintains ATP production (Quinzii et al., 2017) and has been proposed
333 as a growth-related candidate gene (Zhuang et al., 2020). Kleiner and colleagues (Kleiner et al., 2018) found that

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334 an increase of SQOR may prevent oxidative stress by facilitating the antioxidant effects of CoQ10. Reversely, a
335 downregulation of SQOR may reflect deficiency of its coenzyme CoQ10, which in turn alters the sulfide
336 metabolism leading to accumulated H_2S levels and depletion of glutathione, that may cause oxidative damages
337 (Luna-Sánchez et al., 2017; Quinzii et al., 2017; Ziosi et al., 2017). Therefore, the upregulation of SQOR under
338 stress medium treatment could have multiple functions, from metabolising toxic levels of H_2S to restoring both
339 ATP and GSH levels in response to stress communication from a conspecific. Altogether, our results indicate
340 impaired immune and antioxidant responses in embryos exposed to propagated thermal stress.
341
342 **Stress medium and control medium do not induce similar feedback mechanisms**
343 Embryos exposed to the control medium developed slower than control embryos from E3 medium, showing
344 opposite directionality to all other treatments. This opposite directionality was mirrored by the expression patterns
345 of the three investigated genes. The behavioural response to stress medium was also significantly less pronounced
346 than that to control medium, corroborating previous studies where metabolites from undisturbed versus stressed
347 donors induced different responses in several species (Bairos-Novak et al., 2017; Bett et al., 2016). However,
348 activity was much higher in control embryos from E3 medium than in embryos incubated in control medium. This
349 finding may indicate that behavioural activity of zebrafish embryos is tightly controlled by the nature of their
350 chemical environment (vs. relaxed in the absence of any cues), lending additional support to chemical
351 communication as a global change-relevant parameter.
352 Lastly, the similarity in development and behavioural values in combined thermal stress plus stress medium
353 treatments together with a larger effect size indicate that thermal stress and stress medium may excite similar
354 molecular pathways regulating growth rate and behavioural activity, but that these pathways are saturated by single
355 stressors, and cannot be further altered in the combined treatment. Conversely, our gene expression analysis
356 revealed a difference in molecular responses between the two independent factors, thermal stress and stress
357 medium. To better understand these contrasting synergistic vs. independent effects of thermal stress and stress
358 medium, gene expression could be compared at global scale in future work.
359
360 **Conclusion**
361 To conclude, our study indicates that thermally-stressed zebrafish embryos induce a stress response in naive
362 conspecifics that have not been exposed to thermal stress, from molecular to phenotypic level. This extends the
363 concept of stress feedback loops and chemical communication of stress to fluctuating heat stress in a vulnerable
364 life stage of fish. This is important because such stress feedback loops may be widespread and have implications
365 on tipping points of ecosystems facing ongoing and future global change. We suggest further investigation into
366 the identification of the nature of metabolites contained within stress medium, their molecular consequences at an
367 individual level, as well as longer-term consequences for populations and ecosystems.

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376

377 **Conflicts of Interest**

378 The authors declare no conflicts of interest.

379

380 **Ethics approval**

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

382

383 **Consent to participate**

384 All authors consented to participation.

385

386 **Consent for publication**

387 All authors consented to publication.

388

389 **Author contribution**

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

393

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741 **Tables**

742 **Table 1. Effects of thermal stress and stress medium on the growth index of pre-hatching zebrafish embryos.**
743 A two-way ANOVA was used to test the effects of the predictors (thermal stress and stress medium) on changes
744 in growth index. Effect size is computed as Cohen's |d| and interpreted according to thresholds given in
745 Sawilowsky (Sawilowsky, 2009). Effect sizes of significant p-values ($P \leq 0.05$) are shown in bold.
746

Model terms	F statistic	P	|d|	Effect size
Stress Medium	6.291	0.0128	0.29	small
Thermal Stress	75.502	0.0001	1.14	large
Thermal Stress x Stress Medium	7.498	0.0067	1.27	very large

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748 **Table 2. Effects of thermal stress and stress medium on the burst activity percentage of pre-hatching**
749 **zebrafish embryos**. A two-way Scheirer-Ray-Hare test was used to test the effects of the predictors (thermal stress
750 and stress medium) on burst activity percentages across treatments. Effect size is computed as Cohen's |d| and
751 interpreted according to thresholds given in Sawilowsky (Sawilowsky 2009). Effect sizes of significant p-values
752 ($P \leq 0.05$) are shown in bold.
753

Model terms	H statistic	P	|d|	Effect size
Stress Medium	9.3222	0.0023	0.49	small
Thermal Stress	17.0080	0.0001	0.55	medium
Thermal Stress x Stress Medium	1.8193	0.1774	0.99	large

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755 **Table 3. Effect of thermal stress and stress medium on gene expression of pre-hatching zebrafish embryos.**
756 Effects of model terms (thermal stress and stress medium) on the expression of IL-1β, SOD1 and SQOR obtained
757 via moderated t-tests (t-statistic) with *lmfit* and *eBayes* in the *limma* R package. B describes the log-odds of gene
758 expression. Effect size is computed as Cohen's |d| and interpreted according to thresholds given in Sawilowsky
759 (Sawilowsky 2009). Effect sizes of significant p-values ($P \leq 0.05$) are shown in bold.
760

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	1.2	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

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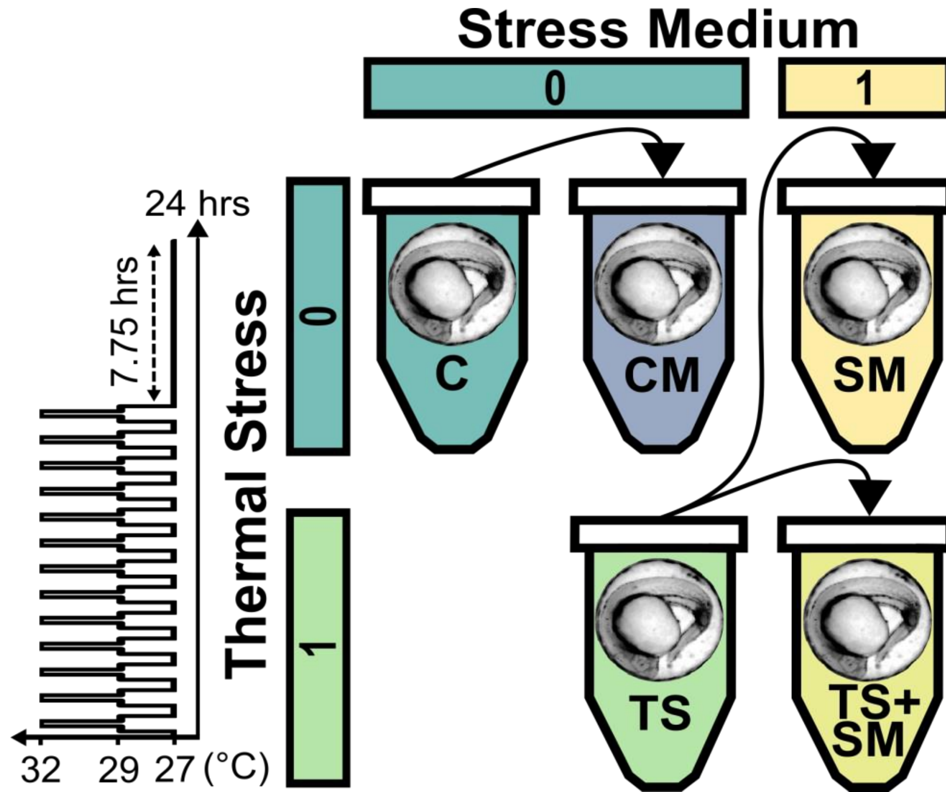
762 **Figures**



763

764

765 **Figure 1. Overview of experimental design.** In a two-way factorial design (represented by 0 and 1 for factor
766 levels, respectively), zebrafish embryos (*Danio rerio*) were exposed to either fluctuating thermal stress (inset
767 diagram) followed by a recovery period (dashed arrow line) or constant control temperature (27°C), in combination
768 with fresh E3 medium or stress medium containing putative stress metabolites produced by one previously
769 thermally-stressed embryo. An additional treatment was incubated at 27°C in a control medium containing
770 metabolites from an embryo previously exposed to control conditions. Plain black arrows indicate medium transfer
771 in which a new embryo was incubated. CM: control medium, C: control, SM: stress medium, TS: thermal stress,
772 TS+SM: thermal stress + stress medium.

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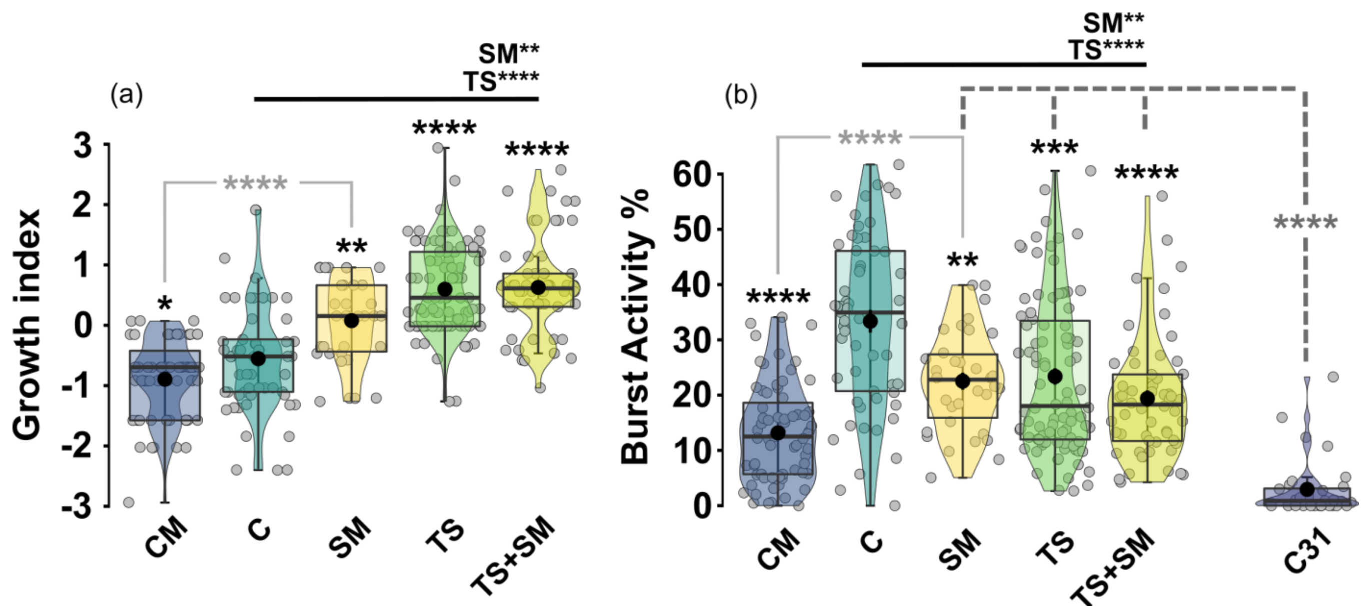

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775 **Figure 2. Thermal stress and stress medium accelerate the growth index (a) and lower the burst activity**
776 **percentage (b) of pre-hatching zebrafish embryos.** CM: control medium (n = 67), C: control (n = 57), SM:
777 stress medium (n = 34), TS: thermal stress (n = 90), TS+SM: thermal stress + stress medium (n = 56), C31: control
778 after 31 hrs of incubation (reaching prim-6 stage). Boxes represent median, 25%-75% quartiles, and whiskers are
779 minimum and maximum values within 1.5 IQR (interquartile range). Grey dots represent individual data points
780 and black dots represent mean values. Effects of predictors (thermal stress and stress medium) were tested with
781 two-factorial tests. Significant predictors are indicated in bold above horizontal black lines with *: $P \leq 0.05$, **: P
782 ≤ 0.01, ***: $P \leq 0.001$, ****: $P \leq 0.0001$. Significance of pairwise post-hoc tests is likewise indicated in asterisks,
783 for treatments against C (bold black), between SM and CM (light grey), or between over-developing treatments
784 (SM, TS, TS+SM) and C31 (dashed bar with dark grey asterisk).

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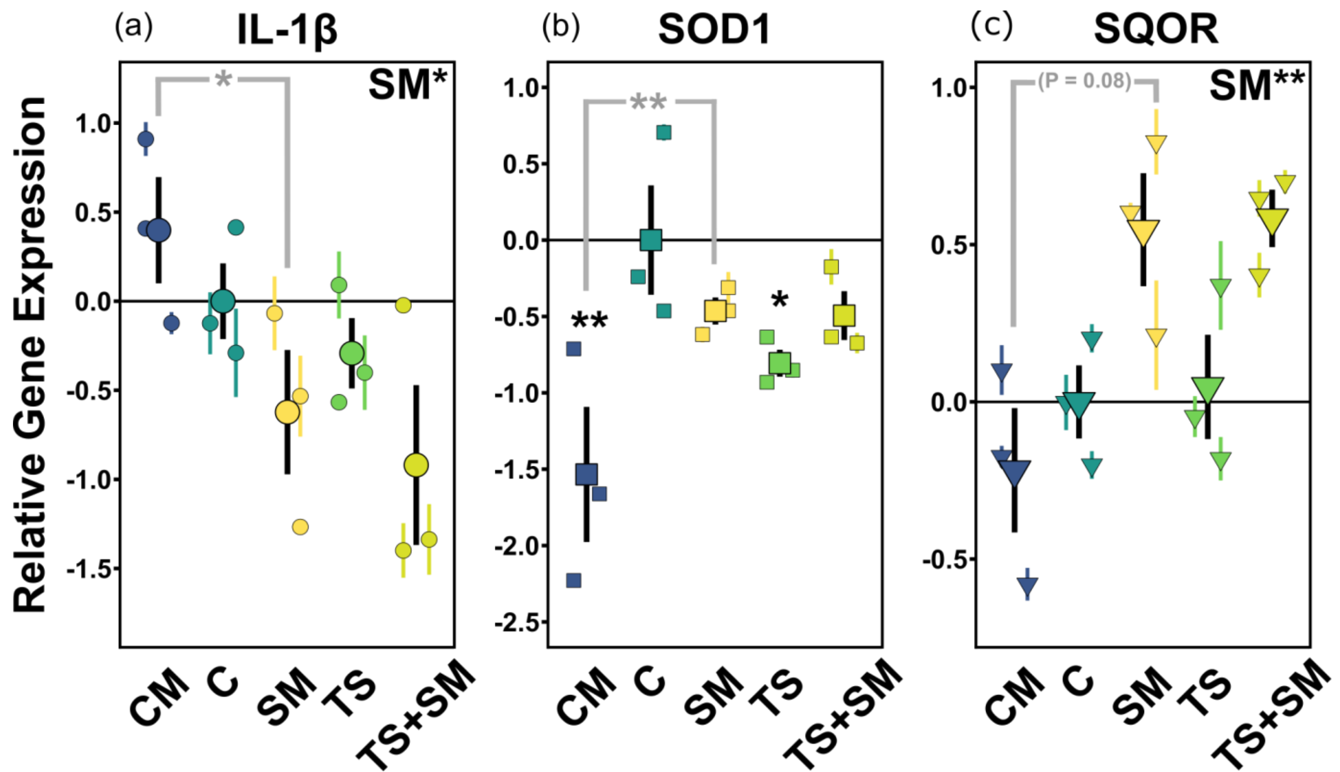

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787 **Figure 3. Stress medium alters stress-related gene expression of zebrafish embryos.** (a) IL-1β (interleukin-
788 1β), involved in immune response. (b) SOD1 (superoxide dismutase 1), involved in antioxidant response. (c)
789 SQOR (sulfide:quinone oxidoreductase), involved in metabolism and antioxidant response. Jittered symbols
790 represent mean $Log_2\ 2^{-\Delta\Delta CT} \pm$ SE (coloured bars) values of each biological replicate. Black symbols represent mean
791 $Log_2\ 2^{-\Delta\Delta CT} \pm$ SE (black bars) values of each biological treatment. Expression is relative to control C (mean values
792 as black horizontal lines). CM: control medium, C: control, SM: stress medium, TS: thermal stress, TS+SM:
793 thermal stress + stress medium. Annotations on top right corners represent the factorial effects of thermal stress
794 (TS) and stress medium (SM) on C, SM, TS, TS+SM. Black asterisks above each mean value only show the
795 pairwise comparisons against the control C. Comparisons between CM and SM are represented in grey by asterisks
796 and dashed horizontal lines. Statistics were computed using moderated t-tests (Limma, Bioconductor, R) with
797 Bonferroni adjustment with *: $P \leq 0.05$, **: $P \leq 0.01$. Sample size: $n = 3$ biological replicates for each treatment,
798 with 60 pooled embryos per each replicate.