

Accepted refereed manuscript of:

Rey S, Moiche Vega AV, Boltana S, Teles M & MacKenzie S (2017)
Behavioural fever in zebrafish larvae, *Developmental and Comparative Immunology*, 67, pp. 287-292.

DOI: [10.1016/j.dci.2016.09.008](https://doi.org/10.1016/j.dci.2016.09.008)

© 2016, Elsevier. Licensed under the Creative Commons Attribution-
NonCommercial-NoDerivatives 4.0 International
<http://creativecommons.org/licenses/by-nc-nd/4.0/>

Accepted Manuscript

Behavioural fever in zebrafish larvae

Sonia Rey, Visila Moiche, Sebastian Boltaña, Mariana Teles, Simon MacKenzie

PII: S0145-305X(16)30285-3

DOI: [10.1016/j.dci.2016.09.008](https://doi.org/10.1016/j.dci.2016.09.008)

Reference: DCI 2726

To appear in: *Developmental and Comparative Immunology*

Received Date: 18 August 2016

Revised Date: 15 September 2016

Accepted Date: 15 September 2016

Please cite this article as: Rey, S., Moiche, V., Boltaña, S., Teles, M., MacKenzie, S., Behavioural fever in zebrafish larvae, *Developmental and Comparative Immunology* (2016), doi: [10.1016/j.dci.2016.09.008](https://doi.org/10.1016/j.dci.2016.09.008).

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1 **Behavioural fever in zebrafish larvae**

2

3 **Sonia Rey^{a1}, Visila Moiche^{a2}, Sebastian Boltaña^{a3}, Mariana Teles^a and Simon**
4 **MacKenzie^{a1*}.**

5

6 ^aInstitut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona,
7 Barcelona, 08193 Bellaterra, Spain

8 ¹Present address: Institute of Aquaculture, University of Stirling, Stirling, Stirlingshire
9 FK9 4LA, UK

10 ²Present address: Fish Facility Resource Room, University College London, Gower
11 Street, London, WC1E 6BT

12 ³Present address: Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary
13 Center for Aquaculture Research (INCAR), University of Concepción, P.O. Box 160-
14 C, Concepción, Chile □

15

16 *Corresponding author

17 Tlf: +44 [0]1786 467991

18 Fax: +44 [0]1786 472133

19

20 E-mail: Simon.MacKenzie@stir.ac.uk

21

22

23

24

25

26

27

28 **Highlights (max 85 characters)**

- 29 • Behavioural fever is a synergic immune response to infection in ectotherms.
- 30 • Zebrafish larvae (*Danio rerio*) select their preferred temperature within a
31 vertical gradient tank.
- 32 • The onset of the behavioural fever response was established at 18-20 dpf.
- 33 • Under an immersion challenge with double-stranded RNA (dsRNA) zebrafish
34 larvae display a behavioural fever response coupled to increased antiviral
35 mRNA transcript abundance.

36 **Abstract**

37 Behavioural fever has been reported in different species of mobile ectotherms
38 including the zebrafish, *Danio rerio*, in response to exogenous pyrogens. In this study
39 we report, to our knowledge for the first time, upon the ontogenic onset of behavioural
40 fever in zebrafish (*Danio rerio*) larvae. For this, zebrafish larvae (from first feeding to
41 juveniles) were placed in a continuous thermal gradient providing the opportunity to
42 select their preferred temperature. The novel thermal preference aquarium was based
43 upon a continuous vertical column system and allows for non-invasive observation of
44 larvae vertical distribution under isothermal (T_R at 28 °C) and thermal gradient
45 conditions (T_{CH} : 28-32°C). Larval thermal preference was assessed under both
46 conditions with or without an immersion challenge, in order to detect the onset of the
47 behavioural fever response. Our results defined the onset of the dsRNA induced
48 behavioural fever at 18-20 days post fertilisation (dpf). Significant differences were
49 observed in dsRNA challenged larvae, which prefer higher temperatures (1-4°C
50 increase) throughout the experimental period as compared to non-challenged larvae. In
51 parallel we measured the abundance of antiviral transcripts; *viperin*, *gig2*, *irf7*, *trim25*
52 and *Mxb* mRNAs in dsRNA challenged larvae under both thermal regimes: T_R and T_{CH} .

53 Significant increases in the abundance of all measured transcripts were recorded under
54 thermal choice conditions signifying that thermo-coupling and the resultant
55 enhancement of the immune response to dsRNA challenge occurs from 18 dpf onwards
56 in the zebrafish. The results are of importance as they identify a key developmental
57 stage where the neuro-immune interface matures in the zebrafish likely providing
58 increased resistance to viral infection.

59 **Keywords**

60 Zebrafish larvae, thermo-preference, behavioural fever, antiviral response, dsRNA
61 challenge, larval development, temperature choice.

62 **1. Introduction**

63 Fever, an ancient defensive reaction from the innate immune system in response
64 to infection, occurs in all groups of vertebrates and some invertebrates (Bicego et al.,
65 2007). Endotherms regulate their body temperature by behavioural and autonomic
66 means by increasing their core body temperature in response to stress or infection
67 (stress induced hyperthermia-SIH and fever). Fever is mediated by endogenous
68 pyrogens such as the prostaglandins or by exogenous pyrogens such as bacterial
69 lipopolysaccharides or viral RNA. The fever response is closely associated with the
70 activation of the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic–
71 adrenal–medullary (SAM) system in mammals or their functional equivalent across the
72 vertebrates leading to the release of adrenocorticotrophic hormone (ACTH) and
73 corticosterone or in fish, cortisol (Adriaan Bouwknecht et al., 2007; Carl V. Gisolfi and
74 Francisco Mora, 2000). Mobile ectotherms such as fish thermoregulate mainly by
75 behavioural means by locating themselves at their preferred optimal temperature in their
76 environment if available. The behavioural fever response is an acute change in the

77 individuals thermal set-point driven by stress (Rey et al., 2015) or by pathogen
78 recognition with a subsequent immune response (Reynolds et al., 1976, Boltana et al.,
79 2013). In ectotherms the fever response is suggested to be mediated by prostaglandins
80 acting at the preoptic area (POAH) of the hypothalamus in the central nervous system
81 (CNS). However the neural pathways responsible for the effector response are still
82 mostly unknown (Bicego et al., 2007; Hamada et al., 2008).

83 Behavioural fever in response to infection has been described in several adult
84 fish species like bluegill (*Lepomis macrochirus*) and goldfish, *Carassius auratus*,,
85 (Reynolds et al., 1978a and b), Mozambique tilapia, *Oreochromis mossambicus*, (Tsai
86 and Hoh, 2012), Nile tilapia, *Oreochromis niloticus*, (Cerqueira et al., 2016) and in
87 response to the proinflammatory cytokine, interleukin 1 beta (IL1 β) in the rainbow trout,
88 *Oncorhynchus mykiss*, (Gräns et al., 2012). In zebrafish, behavioural fever induced by
89 viral infection or dsRNA challenge, promotes extensive and highly specific
90 temperature-dependent changes in the brain transcriptome. These changes, highlighted
91 by a significant increase in antiviral mRNA transcript abundance, promote an
92 abrogation of the viral infection and increased survival (Boltaña et al., 2013). Increased
93 survival to infection has been shown in several studies (Covert and Reynolds, 1977;
94 Elliot et al., 2002; Golovanov, 2006a; Kluger, 1986) suggesting an evolutionary link
95 and important regulatory role for behaviour fever in ectothermic vertebrates.

96 Interestingly, there are few studies available addressing fish larval distribution in
97 thermal gradients (Catalán et al., 2011; Golovanov, 2013; Vollset et al., 2009) although
98 such systems are clearly pertinent to some natural aquatic systems including the native
99 habitat of the zebrafish. On the other hand there is a significant body of research
100 describing the utility of zebrafish for studies of the immune system (Meijer and Spaik,
101 2011; Novoa and Figueras, 2012; Van Der Vaart et al., 2012; Yoder et al., 2002) and the

102 development of the immune system itself (Trede et al., 2004). Innate immune responses
103 have been described earlier than 5 days post fertilisation (dpf) in zebrafish larvae (Dios
104 et al. 2010) although the major maturation events of the complete immune system are
105 described to occur 2-4 weeks post-fertilization (Lam et al., 2004). To our knowledge no
106 studies have reported upon the development of thermal choice behaviour in zebrafish
107 larvae or upon the activation of the immune system under such conditions. A few
108 studies have suggested that fish larvae selecting higher environmental temperatures
109 would exhibit an improved immune performance (Casterlin, 1977; Catalán et al., 2012)
110 however no gene expression data was reported.

111 In this study we firstly report upon thermal preference in zebrafish larvae at
112 different days post fertilization in order to understand dynamic changes in thermal
113 choice behaviour during development. We then identify the emergence of the
114 behavioural fever response to an immersion challenge with dsRNA mimicking a viral
115 infection. Finally we determined how the immune response was enhanced at the gene
116 expression level by measuring selected anti-viral response mRNAs in whole larvae.

117

118 **2. Materials and Methods**

119 *2.1 Animals and rearing conditions*

120 Adult wild-type short fin (WT as defined by ZFIN.org) an unspecified outbred
121 population of zebrafish (*Danio rerio*) were bred and reared in a recirculating aquarium
122 rack system (zfbio labs®) at the aquarium facilities of the IBB (Institut de Biotecnologia
123 i Biomedicina, UAB, Spain). Broodstock were maintained in separated tanks of 25 litres
124 each at $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ water temperature on a 14 L: 10 D photoperiod cycle. A week
125 before breeding they were fed a combination of bloodworm and dry food (zfbio labs®)
126 two or three times per day to improve their body condition. Mass breeding was carried

127 out using six adult males and six adult females, from different breeding stocks, placed
128 together in a small 6 L breeding tank in the afternoon and left overnight. Embryos were
129 collected in the early morning from the breeding tank and transferred to Petri dishes
130 with E3 medium. Embryos and larvae were reared following established protocols
131 (Lawrence, 2007; zfin.org) and fed with an artificial fresh zebrafish larvae diet
132 (zfbiolabs®). At 5 days post fertilization (dpf) when larvae inflated their swim bladders,
133 they were transferred to a 6L tank system and kept at 28°C. At 6 dpf the yolk was
134 mostly depleted and first feeding began. All protocols and animal experiments were
135 approved by the Institutional Animal Care Committee and by the Ethics and Animal
136 Welfare Committee of the Universitat Autònoma de Barcelona, Spain, and adhere to
137 Spanish National and Institutional guidelines and regulations (Dir 2010/63/UE).

138

139 *2.2 Vertical gradient establishment*

140 Experiments were performed using an in house custom-built tank system that
141 consisted of a series of four hollow methacrylate columns (28,5 cm length and 1,2 cm
142 diameter) used as replicate tanks for the experiments. Each tube was filled with treated
143 and filtered water from the aquarium at 28°C. The gradient was established and
144 stabilised using an external water jacket system set at different temperatures. This set-up
145 provided a continuous vertical thermal gradient within the columns (Mean±SD: 32,35°C
146 ± 0,12 - 27,93°C ± 0,26; from top to bottom respectively mimicking natural thermal
147 gradients; see schematic experimental set-up in Fig 1). Water temperature in the vertical
148 column was recorded by a thermal sensors located within different zones of the water
149 column (Thermocouple thermometer 53/54 II, Fluke®). No significant differences were
150 noted in oxygen levels throughout the gradient.

151

152 *2.3 Zebrafish larvae thermopreferendum at different dpf.*

153 Prior to the challenge test, spatial vertical distribution of the zebrafish larvae
154 under normal conditions (non-challenged) in the water column at constant (28°C) and
155 under thermal gradient (27-34°C) was assessed. Under constant temperature we
156 assessed space/area preference and by implementing a more extended gradient for
157 thermal preference we aimed to capture changes in thermal preference during
158 development. Five groups of naïve larvae (n=6 larvae/group) were used. Each group
159 was placed in one of the five gradient columns either at constant temperature or under
160 thermal gradient. Three key different ontogenetic times were selected: 6 dpf, after gas
161 bladder inflation; 13dpf, after yolk sac absorption at the end of early larvae and 24 dpf,
162 close to the end of mid larvae. Distribution of the larvae was assessed by visual
163 instantaneous scan sampling each 15 min during 10 sec for a total of 4 hours (n total=17
164 recorded events per group/ 5 groups = 85 recording events). Larvae were left to
165 acclimatize 30 min before the beginning of the experiment.

166 *2.4 Behavioural fever experiment*

167 Our experimental design was as follows: a total of six replicate groups of 20
168 larvae, (n=120 challenge larvae per 2 thermal conditions+ control non-challenge; at
169 both thermal conditions, N=480; see Fig1) were used for this experiment. Two
170 conditions (thermal restriction or thermal choice: T_R vs T_{CH}) were tested under the same
171 challenge test (dsRNA; 100 µg/ml Poly (I: C)). Larvae were always tested in the same
172 four tubes, used only once, and never fed throughout the experiment (maximum time in
173 gradient 5h). Larvae were introduced into the thermal gradient initially at the same
174 temperature as their acclimation temperature (28°C), at the bottom of the vertical
175 column and habituated for a minimum of 30 minutes to the new environmental
176 conditions. Zebrafish larvae distribution was recorded by an instantaneous visual

177 scanning method every 15 min for 4h (n total=17 recorded events per group/ 6 groups
178 per 2 treatments= 408). Temperature in the testing room was kept at 28°C. When tests
179 were finished whole larvae were carefully collected, instantly frozen in liquid nitrogen
180 and stored at -80°C for posterior molecular analysis.

181 *2.6 RNA isolation, Complementary DNA Synthesis and Quantitative Real-Time* 182 *Polymerase Chain Reaction Assay*

183 RNA was isolated from homogenate pools of whole larvae (20 fish larvae x
184 pool) at similar interval stage of development (18 to 20 dpf) using 1 ml per sample of
185 TriReagent (Molecular Research Centre) following the manufacturer's instructions.
186 RNA quantification was measured with a NanoDrop ND-1000 (Thermo Scientific) and
187 quality verified with the Bioanalyzer 2100 using the 6000 Nano LabChip kit (Agilent
188 Technologies). All RNA integrity number values obtained were >8, indicative of
189 excellent RNA integrity and quality. One microgram of total RNA was used to
190 synthesize complementary DNA (cDNA) with SuperScript IIITM reverse transcriptase
191 (Invitrogen) and oligo-dT primer (Promega). cDNA was used as a template for
192 quantitative real-time polymerase chain reaction assays for the same genes of RT-PCR.
193 Total volume of 20 µl was used, and every reaction contained 500 nM of each
194 amplification primer, 10 µl of iTaqTM Universal SYBR® Green Supermix (BioRad) and
195 5 µl of 1:10 or 1:100 dilution of cDNA (1:1000 for EF-1 α). Controls lacking cDNA
196 were included. Reaction were run in the iCycler iQTM Real-time PCR Detection System
197 (Bio-Rad Laboratories), under the following protocol: 1 cycle of 95 °C for 3 min, 40
198 cycles of 95 °C for 10 sec and 60°C for 30 sec, 70 cycles of 60°C for 10 sec and a
199 melting curve at 60°C. All the samples were run in triplicate. Threshold samples cycle
200 (CT) and calculated a quantification of gene expression relative to untreated controls
201 (Pfaffl, 2001). Values for each sample were expressed as 'fold differences', calculated

202 and normalized to EF-1 α (Elongation factor 1-alpha)(McCurley and Callard, 2008).
203 The relative mRNA abundances of five transcripts representative of the antiviral
204 response (Viperin, Grass Carp Reovirus (GCRV)-induced gene 2: Gig2, Interferon
205 regulatory factor 7: Irf7, tripartite motif containing 25: trim25 and Myxovirus
206 (influenza) resistance B, protein-coding gene: MxB) were compared (see Table 1 for
207 primer sequences and accession numbers).

208 *2.7 Statistical analysis*

209 Statistical analyses were performed using STATISTICA 7.0© (StatSoft, Inc.
210 (2004)) and IBM® SPSS® 17 Statistics v19 for MAC® OS X software. Graphs were
211 plotted using PRISM 6 for Mac OS X software (<http://www.graphpad.com>). Vertical
212 distribution of larvae (at different dpf) under gradient conditions was analysed with a
213 GLM repeated measures ANOVA. Zebrafish larvae distribution along the temperature
214 gradient under a simulated viral infection challenge (control vs. challenged dsRNA
215 larvae) was tested with a non-parametric Mann-Whitney *U* test. Quantitative gene
216 expression data for the 5 different genes studied were examined by a GLM Multivariate
217 ANOVA (MANOVA) for larvae challenged with dsRNA under both different thermal
218 conditions (T_R vs. T_{CH}). Equality of covariance Matrices was tested (Box's Test).
219 Univariate ANOVA followed for each gene specific effect.

220 All data was tested for normality and homogeneity of variances using the
221 Shapiro-Wilk's and Levene's test respectively. Non-normal behavioural data on larval
222 distribution was analysed with non-parametric statistical tests. Gene expression data
223 was \log_{10} transformed to achieve normality and all variances were homogeneous.
224 Significance value was set at $p < 0.05$. Confidence intervals were 95%.

225 **3. Results**

226 3.1 Zebrafish larvae thermopreferendum at different dpf.

227 Zebrafish larvae at constant 28°C (T_R conditions) mainly occupied the surface
228 zone within the vertical column irrespective of age (see supplementary Fig 1: daily
229 rhythms for vertical distribution at constant temperatures for larvae at 6,13 and 24 dpf).
230 In contrast, larvae within the thermal choice environment (T_{CH}), displayed significant
231 changes in thermal preference relative to developmental stage at 6, 13 and 24 dpf.
232 Thermal stratification in the T_{CH} environment clearly influenced vertical distribution
233 and larvae actively sought out preferred temperatures. Larvae from 13 dpf onwards
234 preferred temperatures of 30-31°C (Fig 2) whereas larvae at 6 dpf did not show any
235 discrimination at higher temperature ranges of 32-34°C.

236

237 3.2 Behavioural fever in larvae

238 Using the dsRNA immersion test we were able to identify the onset of
239 behavioural fever at 18-20 dpf. No behavioural fever response was detected before this
240 developmental stage (data not shown). The vertical distribution of larvae throughout the
241 gradient was significantly different between control and dsRNA challenged larval
242 groups (Mann-Whitney U test; $N_1=N_2=120$, at 32°C $U=7821.50$, at 31°C $U=11.452$ and
243 at <30°C $U=8693$, all were $p < 0.001$, Fig. 3). Challenged larvae were located more
244 frequently in the upper zone ($32,35^\circ\text{C} \pm 0,12$) in comparison to sham-treated controls.
245 The latter maintained body temperature in line with the previous thermopreferendum
246 results ($31,10 \pm 0,11^\circ\text{C}$, Fig. 4). Behavioural data residuals on larval distribution across
247 the gradient were not normally distributed even with $\log_{10}(\text{var}+1)$ transformation of
248 the data.

249 3.3 Behavioural fever and gene expression

250 To determine if behavioural fever drives a thermo-coupled modification of the
251 anti-viral response at the mRNA level, as previously observed in adult fish, we
252 compared the dsRNA challenged larvae under temperature gradient (T_{CH} ; thermal
253 choice) and constant temperature (T_R ; thermal restriction) conditions. The mRNA
254 abundance of Viperin, Gig2, Irf7, Trim25 and Mxb viral response transcripts were
255 measured using rtQPCR. Covariance Matrices were equal (Box's Test; $F_{15}=1.289$,
256 $p=0.205$) and the measured transcript abundances highlighted significant group
257 differences between T_{CH} and T_R conditions. The mRNA abundances of the 5 measured
258 transcripts in response to dsRNA were significantly higher in the T_{CH} larvae (Wilks'
259 Lambda; $\Lambda=0.075$, $F_{1,10}=14.88$, $p=0.003$). Differences between Mxb and Gig2 mRNA
260 abundances were the most significant between treatments: one-way ANOVA
261 $F_{1,10}=23.134$ and $F_{1,10}=19.019$, $p<0.001$; see Fig. 5). Irf7 ($F_{1,10}=11.272$, $p<0.01$),
262 Trim25 ($F_{1,10}=10.002$, $p<0.01$) and Viperin mRNA transcripts were also significantly
263 different ($F_{1,10}=5.219$, $p<0.05$).

264 4. Discussion

265 Environmental temperature influences all aspects of an organism's physiology
266 and behaviour, from reproduction to development and growth, and this dynamic
267 interaction impacts upon individual fitness and survival. In mobile ectotherms, such as
268 fish, body temperature closely follows environmental temperature and can only be
269 modified by behavioural means. This behavioural regulation occurs across different
270 temporal scales including daily and seasonal cycles. At a daily/weekly scale our recent
271 studies addressing behavioural and emotional fever responses in adult fish highlight the
272 importance of rapid dynamic changes in thermal preference that impact upon underlying
273 regulation (Boltana et al, 2013, Rey et al, 2015, Cerqueira et al, 2016). This 'thermal
274 choice' experimental model is in stark contrast to the standard experimental approach

275 where fish, as a whole, are kept and challenged under constant temperature regimes that
276 are not similar to that observed in the natural environment.

277 The development of zebrafish larvae has been exceptionally well described and
278 has been a major driver of the rise in use of zebrafish as a universal vertebrate model
279 (Santoriello and Zon, 2012). However to our knowledge there have been no studies
280 addressing thermal choice during the development of zebrafish larvae. A few studies,
281 using different non-model fish species, have evaluated vertical distribution of larval fish
282 in experimental thermal gradients to estimate how a thermal choice can influence larval
283 distribution and how this changes throughout development (Catalán et al., 2011;
284 Golovanov, 2013; Vollset et al., 2009). Our measurements of temperature preference in
285 non-challenged zebrafish larvae are in agreement with these studies highlighting this
286 effect across significant phylogenetic scale. Non-challenged larvae older than 13dpf
287 show a clear preference for 30-31°C even although this higher temperature represents
288 increased oxygen consumption in comparison to the habitual acclimation laboratory
289 temperature of 28.5 °C (López-Olmeda and Sánchez-Vázquez, 2011). The impact of
290 thermally restrictive conditions that is the current practice upon the fitness and welfare
291 of zebrafish has not been addressed. It has previously been suggested that under thermal
292 gradient conditions, larvae prefer temperatures near the upper thermal limit for
293 maximizing growth efficiency (Ehrlich and Muszynski, 1982). Therefore innate thermal
294 preference for higher temperatures in eurythermic fish could decrease from larval to
295 juvenile stages (Magnuson et al., 1979). The underpinning neural circuitry and
296 strategies of thermal choice in vertebrates still remain largely unknown (Hamada et al.,
297 2008) and further research is required to understand how thermal choice is centrally
298 regulated. In this study the ontogenetic effect described highlighted a lack of thermal
299 discrimination (with most larvae going to temperatures > 31°C) in larvae of < 13dpf,

300 suggesting that the thermal sensation network is not fully functional at this
301 developmental stage.

302 Thermal variation is known to have a strong modulatory effect upon the immune
303 response in ectothermic organisms including teleost fish (LeMorvan et al., 1998; Sano
304 et al., 2009; Workenhe et al., 2010). There have been many studies regarding
305 temperature and its impact upon the efficacy of the immune response in fish (Bly and
306 Clem, 1992; Le Morvan et al., 1998; Magnadóttir, 2006; Tort et al., 2003; Watts et al.,
307 2001). There is a general consensus that at higher temperatures, within a species-
308 specific tolerance window, immune responses improve in fish whereas at lower
309 temperatures hamper them (Avunje et al., 2012; Bly and Clem, 1992; LeMorvan et al.,
310 1997; Magnadóttir, 2006). Different responses can be modelled for example in salmon
311 skin across a range of temperatures that highlight the adaptation of the immune response
312 to environmental conditions (Jensen et al., 2015). However it is important to account for
313 temperature effects upon pathogen virulence and the development of disease (Guijarro
314 et al., 2015).

315 It is known that variation in immune responses in ectothermic vertebrates may
316 also be affected by multiple abiotic and intrinsic biological factors including age
317 (Zimmerman et al., 2010). In zebrafish larvae the major maturation events of the
318 immune system occur between 2 and 4 weeks pf (at the larval-juvenile transitory phase)
319 (Lam et al., 2004). This has been suggested to be a developmental strategy based upon
320 the intrinsic link between factors such as nutrient availability, metabolic efficacy,
321 hormonal factors and the developing immune system. Our results in larvae expressing
322 behavioural fever highlight the thermo-coupling of the immune response at 17dpf
323 onwards reflected by the increasingly coordinated dsRNA-TLR3 driven transcriptome
324 activation. T_R larvae showed a lower and generally more scattered response whereas

325 T_{CH} has significantly increased values. Dios et al. (2010) investigated the expression
326 levels of several antiviral transcripts at 28 °C after dsRNA challenge, during larval
327 development in zebrafish. The antiviral response at 28 °C increased during ontogeny
328 until 17 dpf and afterwards decreased in intensity. This result was interpreted as a
329 general trend for more robust responses during the first stages of the development (5-17
330 dpf), at 28 °C (standard laboratory holding temperature condition for zebrafish).
331 According to our data an alternative explanation can be forwarded that suggests that the
332 observed decrease is likely due to the uncoupling of behavioural thermoregulation and
333 the immune response. This would decrease the efficacy of the response, as larvae are
334 unable to express a behavioural fever response. Under T_{CH} conditions, dsRNA
335 challenged larvae express an improved response compared to animals held at T_R , as
336 previously reported in adults (Boltaña et al., 2013). Thus thermocoupling of the
337 immune response exists throughout the life of zebrafish emerging early in the
338 developmental programme.

339

340 **Conclusions**

341 In this study we have demonstrated that zebrafish larvae display shifts in thermal
342 preference when presented with a thermal choice under both normal husbandry and
343 dsRNA stimulated conditions. A significant ontogenetic effect was observed with larvae
344 > 13dpf being able to discriminate between different thermal conditions and actively
345 locating themselves within a specific preferred thermal window. In the absence of a
346 thermal choice larvae migrate vertically to the surface possibly as a conditioned
347 response to food. From 18 - 20 dpf larvae develop a behavioural fever response to
348 dsRNA challenge by modifying their distribution within a thermal gradient column to
349 significantly increase body temperature. This behavioural response is coupled to

350 increase in the anti-viral response demonstrated by increased specific mRNA abundance
351 of key anti-viral factors. The use of thermal gradients by vertebrates during
352 development and throughout their lifecycle is not a novel observation. However the
353 impact of thermal choice upon underpinning molecular responses during development
354 and to pathogens appears to be highly significant. Further studies aiming at different
355 levels of regulation and examining the impact of thermal choice throughout the lifecycle
356 will be essential to understand how ectotherms use thermal gradients to optimize fitness
357 and survival.

358 **Acknowledgments**

359 This study was supported by the Consolider-Ingenio Programme 2010, project
360 CSD2007-0002 funded by the Spanish Ministry of Science and Education, Spain.

361 **References**

- 362
- 363 Avunje, S., Kim, W.-S., Oh, M.-J., Choi, I., Jung, S.-J., 2012. Temperature-dependent
364 viral replication and antiviral apoptotic response in viral haemorrhagic septicaemia
365 virus (VHSV)-infected olive flounder (*Paralichthys olivaceus*). *Fish & Shellfish*
366 *Immunology* 32, 1162–1170.
- 367 Bicego, K.C., Barros, R.C.H., Branco, L.G.S., 2007. Physiology of temperature
368 regulation: comparative aspects. *Comparative biochemistry and physiology. Part*
369 *A, Molecular & integrative physiology* 147, 616–39.
- 370 Bly, J.E., Clem, L.W., 1992. Temperature and teleost immune functions. *Fish &*
371 *Shellfish Immunology* 2, 159–171.
- 372 Boltaña, S., Rey, S., Roher, N., Vargas, R., Huerta, M., Huntingford, F.A., Goetz, F.W.,
373 Moore, J., Garcia-Valtanen, P., Estepa, A., MacKenzie, S., 2013. Behavioural
374 fever is a synergic signal amplifying the innate immune response. *Proceedings of*
375 *the Royal Society B: Biological Sciences* 280 .
- 376 Bouwknecht, A.J., Olivier, B., Paylor, R.E., 2007. The stress-induced hyperthermia
377 paradigm as a physiological animal model for anxiety: A review of
378 pharmacological and genetic studies in the mouse. *Neuroscience and Biobehavioral*
379 *Reviews* 31, 41–59.
- 380 Casterlin, M.E.R.W.W., 1977. Behavioral fever in anuran amphibian larvae. *Methods*
381 20, 593–596.
- 382 Catalán, I. a., Vollset, K.W., Morales-Nin, B., Folkvord, a., 2011. The effect of

- 383 temperature gradients and stomach fullness on the vertical distribution of larval
384 herring in experimental columns. *Journal of Experimental Marine Biology and*
385 *Ecology* 404, 26–32.
- 386 Catalán, T.P., Niemeyer, H.M., Kalergis, A.M., Bozinovic, F., 2012. Interplay between
387 behavioural thermoregulation and immune response in mealworms. *Journal of*
388 *Insect Physiology* 58, 1450–1455.
- 389 Cerqueira M, Rey S, Silva T, Featherstone Z, Crumlish M, MacKenzie S. Thermal
390 preference predicts animal personality in Nile tilapia *Oreochromis niloticus*. *J*
391 *Anim Ecol*. 2016 Sep;85(5):1389-400
- 392 Covert, J.B., Reynolds, W.W., 1977. Survival value of fever in fish. *Nature* 267, 43–45.
- 393 Dios, S., Romero, A., Chamorro, R., Figueras, A., Novoa, B., 2010. Effect of the
394 temperature during antiviral immune response ontogeny in teleosts. *Fish and*
395 *Shellfish Immunology* 29, 1019–1027.
- 396 Ehrlich, K.F., Muszynski, G., 1982. Effects of temperature on interactions of
397 physiological and behavioural capacities of larval California grunion: Adaptations
398 to the planktonic environment. *Journal of Experimental Marine Biology and*
399 *Ecology* 60, 223–244.
- 400 Elliot, S.L., Blanford, S., Thomas, M.B., 2002. Host-pathogen interactions in a varying
401 environment: temperature, behavioural fever and fitness. *Proceedings. Biological*
402 *sciences / The Royal Society* 269, 1599–607.
- 403 Gisolfi, C.V and Mora, F. *The Hot Brain*. The MIT Press ISBN: 9780262515344
- 404 Golovanov, V.K., 2006. The ecological and evolutionary aspects of thermoregulation
405 behavior on fish. *Journal of Ichthyology* 46, S180–S187.
- 406 Golovanov, V.K., 2013. Ecophysiological patterns of distribution and behavior of
407 freshwater fish in thermal gradients. *Journal of Ichthyology* 53, 252–280.
- 408 Gräns, A., Rosengren, M., Niklasson, L., Axelsson, M., 2012. Behavioural fever boosts
409 the inflammatory response in rainbow trout *Oncorhynchus mykiss*. *Journal of Fish*
410 *Biology* 81, 1111–1117.
- 411 Guijarro, J.A., Cascales, D., García-Torrico, A.I., García-Domínguez, M., Méndez, J.,
412 2015. Temperature-dependent expression of virulence genes in fish-pathogenic
413 bacteria. *Frontiers in microbiology* 6, 700.
- 414 Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., Garrity,
415 P.A., 2008. An internal thermal sensor controlling temperature preference in
416 *Drosophila*. *Nature* 454, 217–20.
- 417 Jensen, L.B., Boltana, S., Obach, A., McGurk, C., Waagbø, R., Mackenzie, S., 2015.
418 Investigating the underlying mechanisms of temperature-related skin diseases in
419 Atlantic salmon, *Salmo salar* L., as measured by quantitative histology, skin
420 transcriptomics and composition. *Journal of Fish Diseases* 38, 977–992.
- 421 Kluger, M.J., Ph, D., 1986. Is Fever Beneficial ? *Acta Physiologica Scandinavica* 59,
422 89–95.
- 423 Lam, S.H., Chua, H.L., Gong, Z., Lam, T.J., Sin, Y.M., 2004. Development and
424 maturation of the immune system in zebrafish, *Danio rerio*: A gene expression
425 profiling, in situ hybridization and immunological study. *Developmental and*
426 *Comparative Immunology* 28, 9–28.

- 427 Lawrence, C., 2007. The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture*.
- 428 Le Morvan, C., Troutaud, D., Deschaux, P., 1998. Differential effects of temperature on
429 specific and nonspecific immune defences in fish. *The Journal of experimental*
430 *biology* 201, 165–168.
- 431 LeMorvan, C., Clerton, P., Deschaux, P., Troutaud, D., 1997. Effects of environmental
432 temperature on macrophage activities in carp. *Fish & Shellfish Immunology* 209–
433 212.
- 434 LeMorvan, C.L.E., Troutaud, D., Deschaux, P., 1998. DIFFERENTIAL EFFECTS OF
435 TEMPERATURE ON SPECIFIC AND NONSPECIFIC IMMUNE DEFENCES
436 IN FISH 168, 165–168.
- 437 López-Olmeda, J.F., Sánchez-Vázquez, F.J., 2011. Thermal biology of zebrafish (*Danio*
438 *rerio*). *Journal of Thermal Biology*.
- 439 Magnadóttir, B., 2006. Innate immunity of fish (overview). *Fish and Shellfish*
440 *Immunology* 20, 137–151.
- 441 Magnuson, J.J., Crowder, L.B., Medvick, P.A., 1979. Temperature as an ecological
442 resource. *Integrative and Comparative Biology* 19, 331–343.
- 443 McCurley, A.T., Callard, G. V., 2008. Characterization of housekeeping genes in
444 zebrafish: male-female differences and effects of tissue type, developmental stage
445 and chemical treatment. *BMC molecular biology* 9, 102.
- 446 Meijer, A.H., Spaink, H.P., 2011. Host-pathogen interactions made transparent with the
447 zebrafish model. *Current drug targets* 12, 1000–17.
- 448 Novoa, B., Figueras, A., 2012. Zebrafish: model for the study of inflammation and the
449 innate immune response to infectious diseases. *Adv Exp Med Biol* 946, 253–275.
- 450 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time
451 RT-PCR. *Nucleic acids research* 29, e45.
- 452 Rey, S., Huntingford, F.A., Boltana, S., Vargas, R., Knowles, T.G., Mackenzie, S.,
453 2015. Fish can show emotional fever: stress-induced hyperthermia in zebrafish.
454 *Proc R Soc B* 282, 20152266–.
- 455 Reynolds, W.W., Casterlin, M.E., Covert, J.B., 1976. Behavioural fever in teleost
456 fishes. *Nature* 259, 41–42.
- 457 Reynolds, W.W., Casterlin, M.E., Covert, J.B., 1978. Febrile responses of bluegill
458 (*Lepomis macrochirus*) to bacterial pyrogens. *Journal of Thermal Biology* 3, 129–
459 130.
- 460 Sano, M., Ito, T., Matsuyama, T., Nakayasu, C., Kurita, J., 2009. Effect of water
461 temperature shifting on mortality of Japanese flounder *Paralichthys olivaceus*
462 experimentally infected with viral hemorrhagic septicemia virus. *Aquaculture* 286,
463 254–258.
- 464 Santoriello, C., Zon, L.I., 2012. Hooked! modeling human disease in zebrafish. *Journal*
465 *of Clinical Investigation*.
- 466 Tort, L., Balasch, J.C., Mackenzie, S., 2003. Fish immune system. A crossroads
467 between innate and adaptive responses. *Immunologia* 22, 277–286.
- 468 Trede, N.S., Langenau, D.M., Traver, D., Look, A.T., Zon, L.I., 2004. The use of
469 zebrafish to understand immunity. *Immunity*.
- 470 Tsai, C.-L., Hoh, K.-H., 2012. Short Note. *Photographies* 5, 247–247.

- 471 Van Der Vaart, M., Spaink, H.P., Meijer, A.H., 2012. Pathogen recognition and
472 activation of the innate immune response in zebrafish. *Advances in Hematology*.
- 473 Vollset, K.W., Fiksen, Folkvord, a., 2009. Vertical distribution of larval cod (*Gadus*
474 *morhua*) in experimental temperature gradients. *Journal of Experimental Marine*
475 *Biology and Ecology* 379, 16–22.
- 476 Watts, M., Munday, B.L., Burke, C.M., 2001. Immune responses of teleost fish.
477 *Australian veterinary journal* 79, 570–574.
- 478 Workenhe, S.T., Rise, M.L., Kibenge, M.J.T., Kibenge, F.S.B., 2010. The fight between
479 the teleost fish immune response and aquatic viruses. *Molecular Immunology* 47,
480 2525–2536.
- 481 Yoder, J.A., Nielsen, M.E., Amemiya, C.T., Litman, G.W., 2002. Zebrafish as an
482 immunological model system. *Microbes and Infection*.
- 483 Zimmerman, L.M., Paitz, R.T., Vogel, L. a, Bowden, R.M., 2010. Variation in the
484 seasonal patterns of innate and adaptive immunity in the red-eared slider
485 (*Trachemys scripta*). *The Journal of experimental biology* 213, 1477–1483.

486

487

488

489 **Tables**

490 **Table 1** Primer sequences designed for qPCR analyses of selected mRNA transcripts.
491 EF-1a was used as a housekeeping control.

492

493 **Figure captions**

494 **Fig. 1.** Vertical gradient tank design: Four independent methacrylate columns, 28,5 cm
495 length and 1,2 cm diameter, were filled with filtered water and heated and cooled by
496 externally running water through the upper, middle and bottom exterior compartments.
497 The columns were divided into 5 zones (Z1-5) each representing a 1⁰C step in mean
498 temperature in the gradient. Each column holds n=20 larvae.

499 **Fig. 2** Mean distribution of larvae at differential developmental stages (6, 13 and 24dpf)
500 in the thermal gradient over time (4 hours). Under gradient conditions, 6dpf larvae do
501 not show temperature discrimination whereas larvae >13dpf show preference for
502 temperatures $\approx 30^{\circ}\text{C}$ (repeated measures ANOVA, $F(8,1008)=35.296$; $p<0.0001$).

503 **Fig. 3** Frequency of occupation for zebrafish larvae along the thermal gradient
504 challenged with dsRNA (poly (I:C), 100 µg/ml) or untreated control. Mann-Whitney *U*
505 test; $p < 0.001$ (Mean \pm SD, *** $p < 0.001$).

506 **Fig. 4** Behavioural fever in dsRNA-challenged zebrafish larvae. Thermal zone
507 occupation (32°C) for zebrafish larvae along the thermal gradient challenged with
508 dsRNA (poly (I:C), 100 µg/ml) or control untreated. Mann-Whitney *U* test;
509 $p < 0.001$ (Mean \pm SD, ** $p < 0.01$).

510 **Fig. 5** Comparison of the abundance of five antiviral mRNA transcripts after 4h post-
511 dsRNA challenge (poly (I:C), 100 µg/ml), T_{Ch} (28-32 °C) versus T_R (28 °C) in pooled
512 zebrafish larvae ($n = 20$ larvae per pool) (GLM MANOVA, ** $p < 0.01$). Values shown
513 on individual columns are mRNA relative abundance ratios (Mean \pm SD, *GLM one-way*
514 *ANOVA*, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

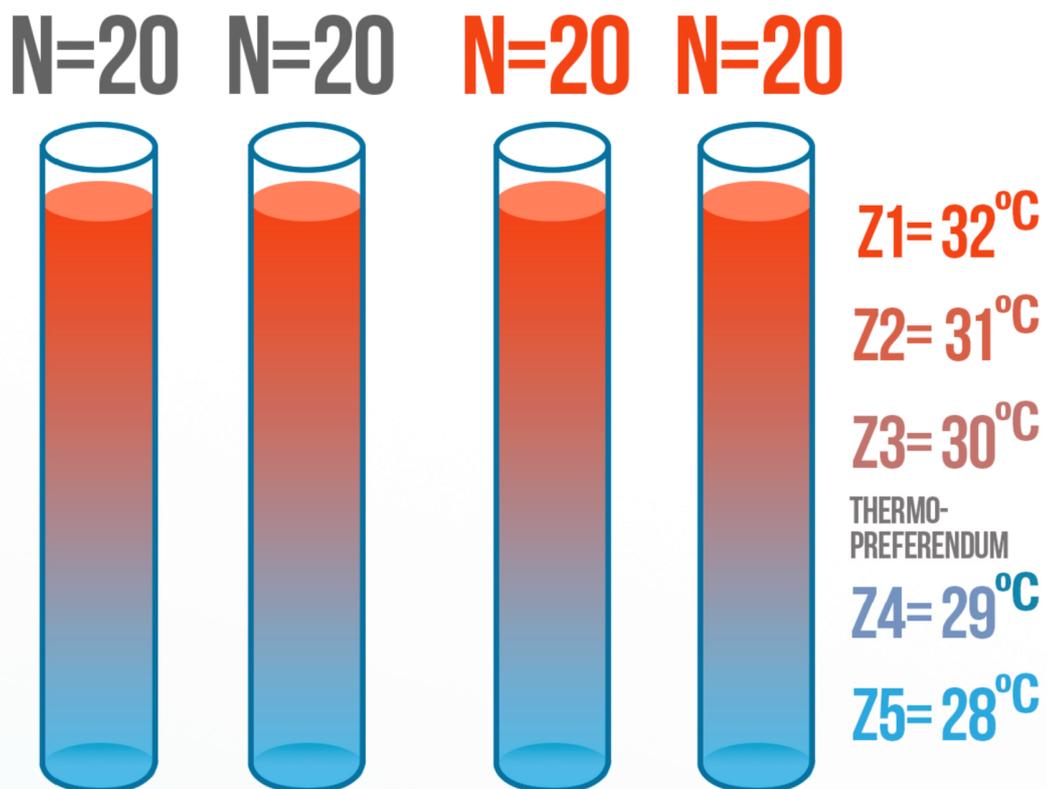
515 **SFig. 1.** Mean distribution of larvae at differential developmental stages (6, 13 and
516 24dpf) at constant temperature over time (4 hours). At constant conditions all zones
517 were at the same temperature (28°C) and larvae were mostly at the water surface.

518

Primer	Sequence (5'-3')	Accession number
Viperin F	GCTGAAAGAAGCAGGAATGG	EF014961.1
Viperin R	AAACTGGAAGACCTTCCAA	
Mxb(b) F	AATGGTGATCCGCTATCTGC	AJ544824.2
Mxb(b) R	TCTGGCGGCTCAGTAAGTTT	
IRf7(a) F	AGGCAGTTCAACGTCAGCTACCAT	NM_200677.1
IRf7(a) R	TTCCACCAAGTTGAGCAATTCCAG	
Trim25 F	TGCATCAAGAGCTGACACAA	XM_001337964.4
Trim25 R	GTGAAGTGAAGCTGGGAACA	
Gig2 F	AGGGTACGACACTGCCTGGT	NM_001245991.2
Gig2 R	AGGGTCACCAAAGCCACAAT	
EF-1 α F	CTTCTCAGGCTGACTGTGC	AY422992
EF-1 α R	CCGCTAGCATTACCTCC	

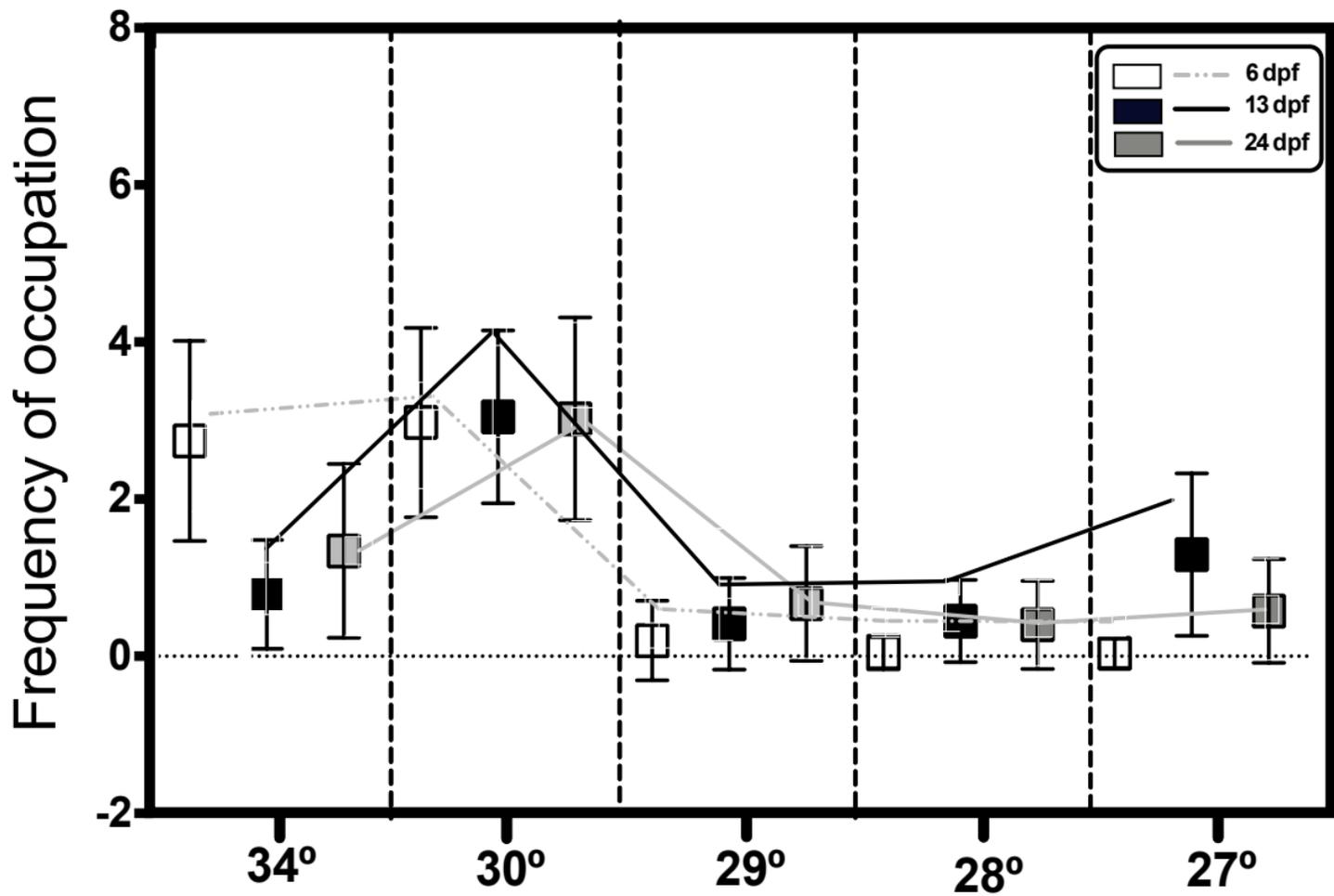
Table 1. Primer sequences designed for qPCR analysis of selected genes. EF-1 α was chosen as housekeeping gene.

ACCEPTED



Vertical thermal gradient

ACCEPTED



Frequency of occupation

