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Behavioural fever in zebrafish larvae

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Highlights (max 85 characters)

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

Abstract

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

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

Keywords

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

1. Introduction

Fever, an ancient defensive reaction from the innate immune system in response to infection, occurs in all groups of vertebrates and some invertebrates (Bicego et al., 2007). Endotherms regulate their body temperature by behavioural and autonomic means by increasing their core body temperature in response to stress or infection (stress induced hyperthermia-SIH and fever). Fever is mediated by endogenous pyrogens such as the prostaglandins or by exogenous pyrogens such as bacterial lipopolysaccharides or viral RNA. The fever response is closely associated with the activation of the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic–adrenal–medullary (SAM) system in mammals or their functional equivalent across the vertebrates leading to the release of adrenocorticotropic hormone (ACTH) and corticosterone or in fish, cortisol (Adriaan Bouwknecht et al., 2007; Carl V. Gisolfi and Francisco Mora, 2000). Mobile ectotherms such as fish thermoregulate mainly by behavioural means by locating themselves at their preferred optimal temperature in their 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 (Il1β) 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 Spaink,
101 2011; Novoa and Figueras, 2012; Van Der Vaart et al., 2012; Yoder et al., 2002) and the

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

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

2. Materials and Methods

2.1 Animals and rearing conditions

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

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

2.2 Vertical gradient establishment

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

2.3 Zebrafish larvae thermopreferendum at different dpf.

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

2.4 Behavioural fever experiment

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

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

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

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

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

2.7 Statistical analysis

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

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

3. Results

226 *3.1 Zebrafish larvae thermopreferendum at different dpf.*

227 Zebrafish larvae at constant 28ºC (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 30-31ºC (Fig 2) whereas larvae at 6 dpf did not show any
235 discrimination at higher temperature ranges of 32-34ºC.

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; N1=N2=120, at 32ºC U=7821.50, at 31ºC U=11.452 and
243 at <30ºC U=8693, all were $p < 0.001$, Fig. 3). Challenged larvae were located more
244 frequently in the upper zone (32,35ºC ± 0,12) in comparison to sham-treated controls.
245 The latter maintained body temperature in line with the previous thermopreferendum
246 results (31,10 ± 0,11ºC, Fig. 4). Behavioural data residuals on larval distribution across
247 the gradient were not normally distributed even with log10 (var+1) transformation of
248 the data.

249 *3.3 Behavioural fever and gene expression*

To determine if behavioural fever drives a thermo-coupled modification of the anti-viral response at the mRNA level, as previously observed in adult fish, we compared the dsRNA challenged larvae under temperature gradient (T_{CH}; thermal choice) and constant temperature (T_R; thermal restriction) conditions. The mRNA abundance of Viperin, Gig2, Irf7, Trim25 and Mxb viral response transcripts were measured using rtQPCR. Covariance Matrices were equal (Box's Test; F_{15}=1.289, p=0.205) and the measured transcript abundances highlighted significant group differences between T_{CH} and T_R conditions. The mRNA abundances of the 5 measured transcripts in response to dsRNA were significantly higher in the T_{CH} larvae (Wilks' Lambda; $\Lambda =$ 0,075, $F_{1,10}$=14.88, p=0.003). Differences between Mxb and Gig2 mRNA abundances were the most significant between treatments: one-way ANOVA $F_{1,10}$=23.134 and $F_{1,10}$,=19.019, p<0.001; see Fig. 5). Irf7 ($F_{1,10}$,=11.272, p<0.01), Trim25 ($F_{1,10}$,=10.002, p<0.01) and Viperin mRNA transcripts were also significantly different ($F_{1,10}$,=5.219, p<0.05).

4. Discussion

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

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

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

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

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

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

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

Conclusions

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

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

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Tables

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

Figure captions

Fig. 1. Vertical gradient tank design: Four independent methacrylate columns, 28,5 cm length and 1,2 cm diameter, were filled with filtered water and heated and cooled by externally running water through the upper, middle and bottom exterior compartments. The columns were divided into 5 zones (Z1-5) each representing a 1^0C step in mean temperature in the gradient. Each column holds n=20 larvae.

Fig. 2 Mean distribution of larvae at differential developmental stages (6, 13 and 24dpf) in the thermal gradient over time (4 hours). Under gradient conditions, 6dpf larvae do not show temperature discrimination whereas larvae >13dpf show preference for temperatures ≈ 30ºC (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 ± SD, $^{***}p < 0.001$).

506 **Fig. 4** Behavioural fever in dsRNA-challenged zebrafish larvae. Thermal zone
507 occupation (32^{o}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 ± SD, $^{**}p < 0.01$).

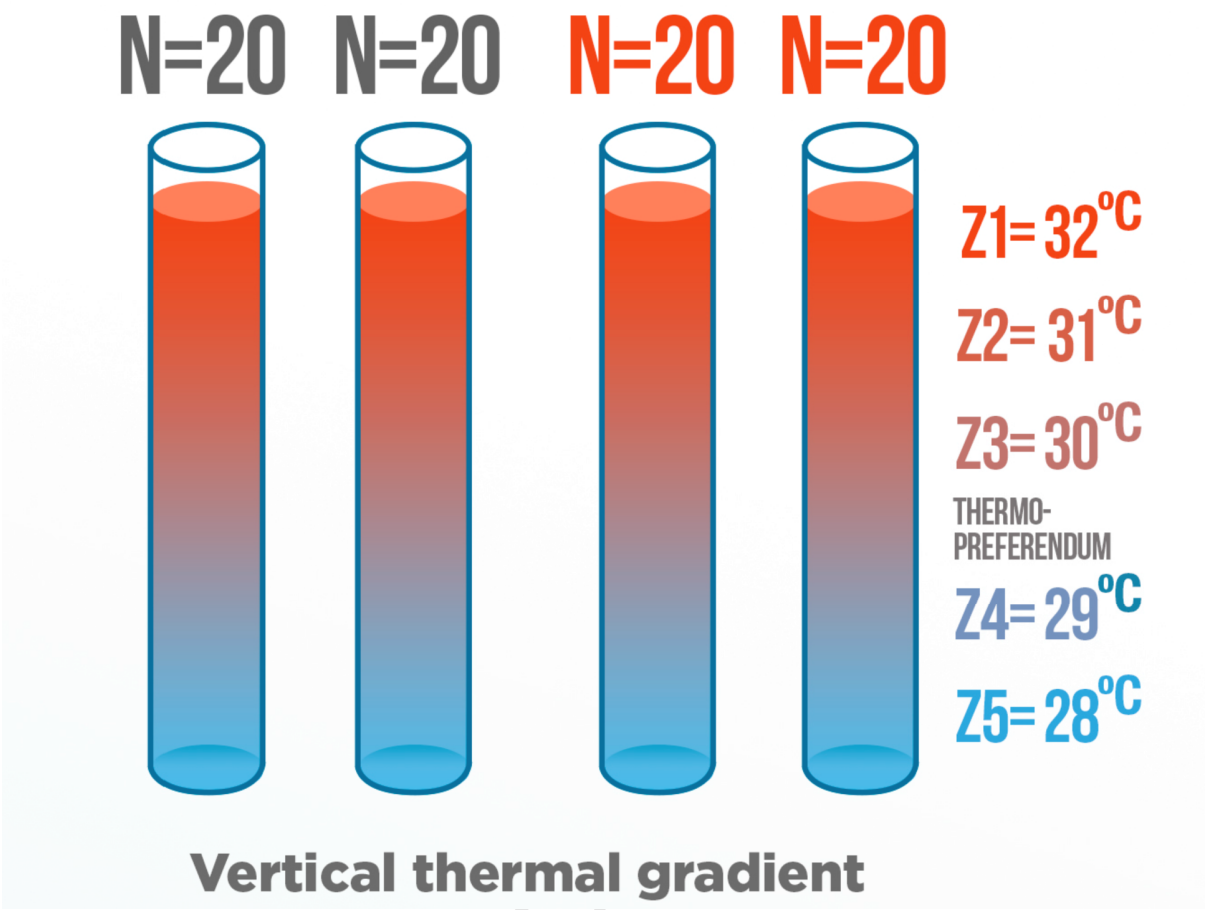
510 **Fig. 5** Comparison of the abundance of five antiviral mRNA transcripts after 4h post-
511 dsRNA challenge (poly (I:C), 100 µg/ml), T_{Ch} (28-32 ºC) versus T_R (28 ºC) in pooled
512 zebrafish larvae (n = 20 larvae per pool) (GLM MANOVA, $^{**}p < 0.01$). Values shown
513 on individual columns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

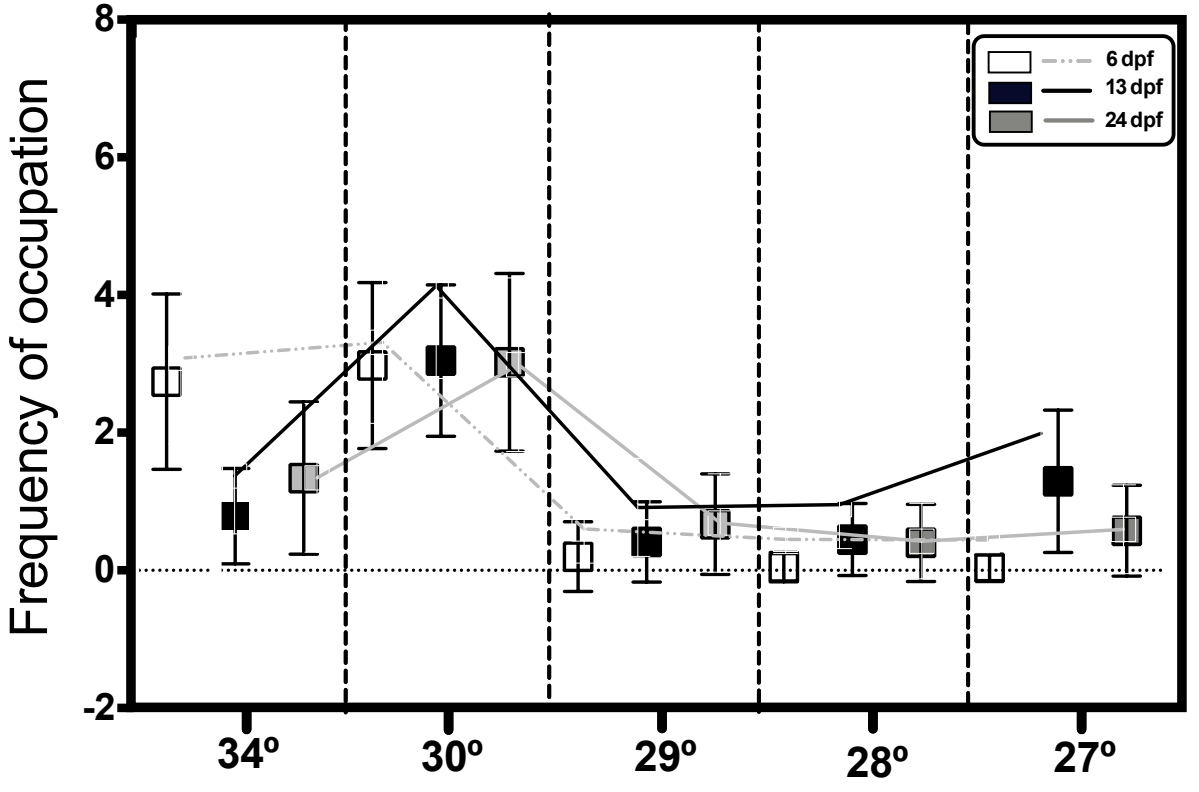
515 **SFig. 1.** Mean distribution of larvae at differential developmental stages (6, 13 and
516 24dpf) at constant temperature over time (4 hours). At constant conditions all zones
517 were at the same temperature (28ºC) and larvae were mostly at the water surface.

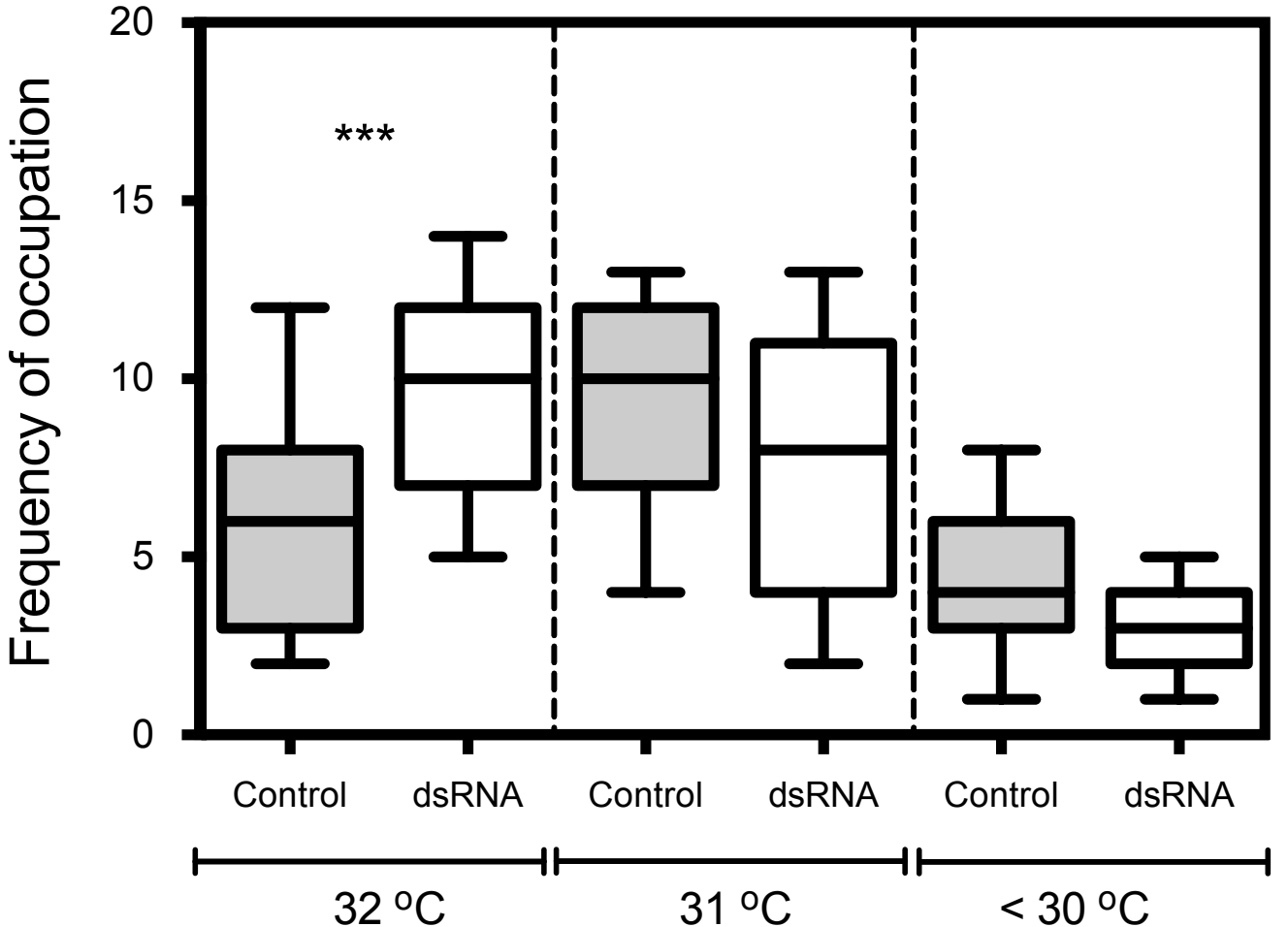
518

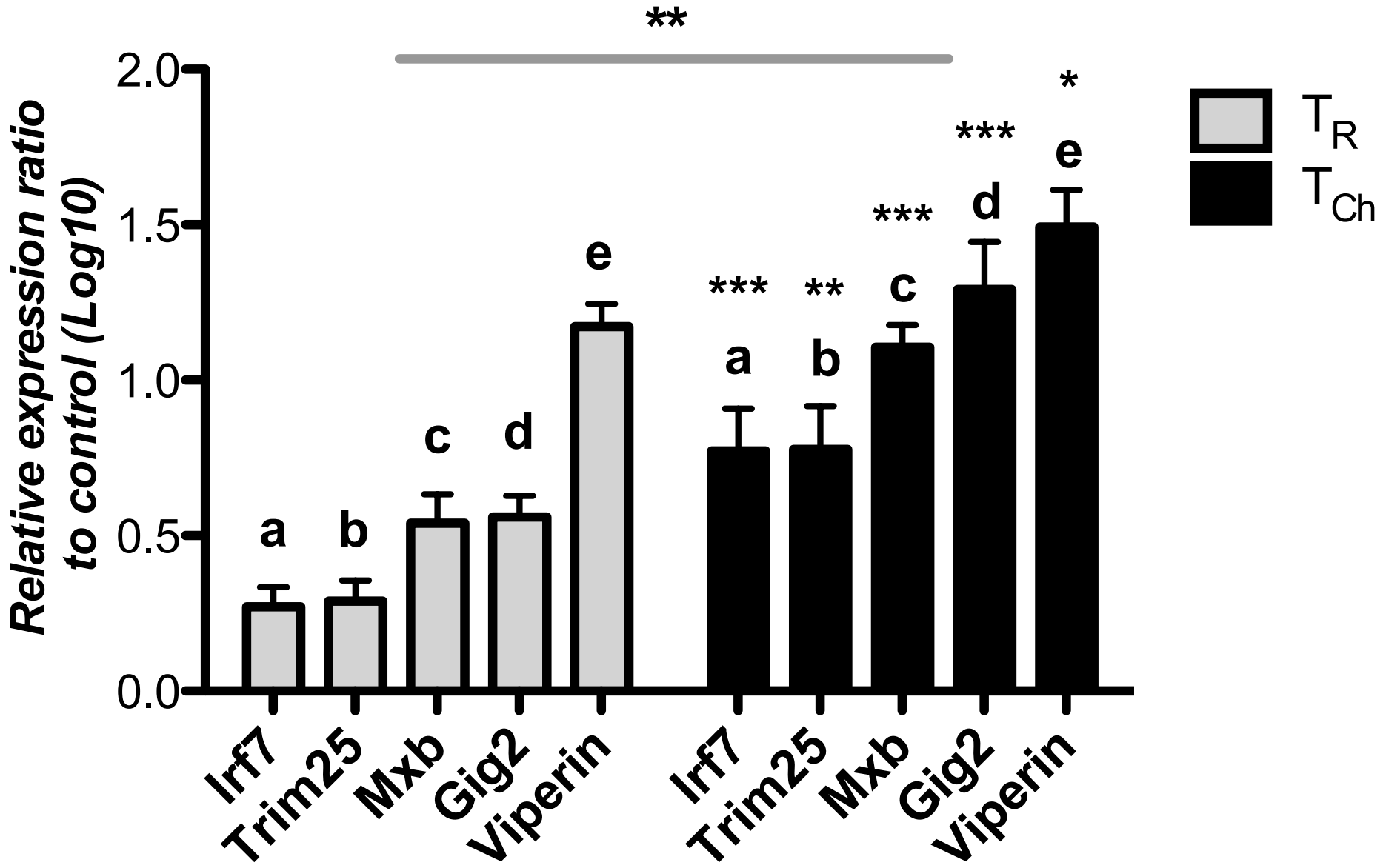
Primer	Sequence (5'-3')	Accession number
Viperin F Viperin R	GCTGAAAGAAGCAGGAATGG AAACACTGGAAGACCTTCCAA	EF014961.1
Mxb(b) F Mxb(b) R	AATGGTGATCCGCTATCTGC TCTGGCGGCTCAGTAAGTTT	AJ544824.2
IRf7(a) F IRf7(a) R	AGGCAGTTCAACGTCAGCTACCAT TTCCACCAAGTTGAGCAATTCCAG	NM_200677.1
Trim25 F Trim25 R	TGCATCAAGAGCTGACACAA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

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


N=20
N=20
N=20
N=20
Z1= 32°C
Z2= 31°C
Z3= 30°C
THERMO-
PREFERENDUM
Z4= 29°C
Z5= 28°C
Vertical thermal gradient


Frequency of occupation
8
6
4
2
0
-2
6 dpf
13 dpf
24 dpf
34º
30º
29º
28º
27º


Frequency of occupation
20
15
10
5
0
***
Control
dsRNA
Control
dsRNA
Control
dsRNA
32 ºC
31 ºC
< 30 ºC


**
Relative expression ratio to control (Log10)
2.0
1.5
1.0
0.5
0.0
a
b
c
d
e
***
**
***
***
*
a
b
c
d
e
Irf7
Trim25
Mxb
Gig2
Viperin
Irf7
Trim25
Mxb
Gig2
Viperin
T_R
T_Ch