EFFECT OF THE TEMPERATURE DURING ANTIVIRAL IMMUNE RESPONSE ONTOGENY IN TELEOSTS

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ABSTRACT

Zebrafish were used to investigate the expression levels of several antiviral and inflammatory genes (IL-1β, iNOS, TNF-α, TLR3, IFN-I, IFNγ, IRF3, MDA-5, Mx) constitutively and after viral stimulation during early development. We also determined how their expression was affected by changes in the temperature. The antiviral genes were almost completely inhibited at 15 ºC with the exception of TLR3. In contrast, IL-1β, iNOS and TNF-α expression was not obviously different between the two temperatures. At 15 ºC, most of the genes examined did not differ following stimulation with poly I:C or viral hemorrhagic septicemia virus (VHSV). However, at 28 ºC, all of the genes showed significant differences in at least some of the sampling points after poly I:C treatment with the largest differences observed for Mx. Mx expression in adult zebrafish was not significantly altered by temperature and poly I:C treatment led to a smaller increase in gene expression when compared to larval Mx levels. Thus, Mx seems to play an important role in viral immunity in larvae, when the adaptive immune response is not fully functional.

Keywords: zebrafish, innate immunity, temperature, ontogeny, proinflammatory, antiviral, poly I:C, VHSV

1. INTRODUCTION

The cellular and humoral components of the mammalian immune system develop sequentially in the fetus, leaving organisms at this developmental stage susceptible to invading bacteria and viruses [1]. Although immunocompetent cells mature rather early in embryogenesis [2], the immune system is ineffective at the embryonic and early postnatal periods [3]. Further, there is considerable evidence in mammals that the immune system of neonates is functionally different from that of adults [4-10].

Fish [11,12] and amphibian [13] immune systems include almost the full repertoire of lymphoid organs and immune cell types as those found in mammals. However, unlike mammals, the early development of the fish and amphibian immune systems occur in the open environment. Indeed, *Xenopus laevis* larvae hatch 2 days following fertilization and zebrafish (*Danio rerio*) hatch 2-3 days post-fertilization. As such, their immune systems are under considerable pressure to develop quickly and produce a heterogeneous immune repertoire [13-15].

The zebrafish has recently emerged as a model for the study of vertebrate immunity, revealing a fully developed innate and adaptive immune system [16-27]. Interestingly, there is a clear temporal separation between the innate and adaptive immune systems in zebrafish. The innate immune system is detectable and active at day 1 of zebrafish embryogenesis [28-30]. In contrast, the adaptive immune system is not morphologically and functionally mature until 4-6 weeks post-fertilization of the egg (wpf) [17,31-33]. This temporal separation allows for the *in vivo* study of the vertebrate innate immune response independent of adaptive immunity [34].

In this work, the zebrafish was used to investigate the constitutive expression patterns of several proinflammatory and antiviral genes related to the innate immune

response, including IL-1β, iNOS, TNF-α, TLR3, IFN-I, IFNγ, IRF3, MDA-5 and Mx. The expression of these genes was also examined after stimulation with poly I:C, a TLR3 ligand, during early larval development. Finally, whether proinflammatory and antiviral gene expression is affected by temperature was determined.

2. MATERIAL AND METHODS

2.1 Animals

Adult, embryo and larval zebrafish were obtained from our experimental facility where zebrafish are cultured following established protocols [35,36] (also see http://zfin.org/zf_info/zfbook/zfbk.html).

2.2 Experimental treatments

Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics. Poly I:C was used to mimic a viral infection during different experimental challenges. In addition, VHSV (strain 0771) was used to infect larvae at low temperature. Several spawnings from the same stock were induced to obtain larvae at different days post-fertilization (dpf) from 2 dpf to 29 dpf at 3-day intervals. Different numbers of embryos or fish were used for each age group due to the differences in size to assure a minimum RNA amount (always 1 microgram) for cDNA transcription. Two biological replicates with 10-15 animals each (for individuals ranging from 2-14 dpf) or 6-8 animals each (for individuals ranging from 17-29 dpf) were treated with poly I:C (0.1 mg/mL, Sigma) at either 15 or 28 ºC by bath. Animals were maintained at each temperature for 24 hours. The same protocol was used for VHSV infection ($5x10^9$ $TCID_{50}$/ml) at 15 ºC. Two duplicate groups were used as control fish for both poly I:C stimulation and VHSV infection.

In another set of experiments, three groups of 24 adult zebrafish were anesthetized with MS-222 (Tricaine methanesulfonate, Argent Chemical Laboratories, USA) and intraperitoneally injected with 10 μL of 1 mg/mL poly I:C solution. The first and second groups were maintained 24 hours at 15 and 28 ºC, respectively, post-stimulation as conducted in larvae. The third group was allowed to acclimatize for 1 week at 15 ºC before stimulation and maintained for an additional 24 h at 15 ºC. This acclimation was not possible in larvae because of the high mortality observed when they are maintained at temperatures below 28 ºC for long periods of time. Twenty-four fish per group were injected with PBS and used as controls. Head kidneys were sampled at 3, 6 and 24 hours post-stimulation.

2.3 RNA isolation and cDNA transcription

Larvae were pooled together after 24 hours post-stimulation (hps) or post-infection (hpi) in 500 μL of TRIzol reagent (Invitrogen) for each sampling point, treatment and temperature and preserved at -80 ºC until use. In the case of adults, zebrafish were killed by an anesthetic overdose and the kidneys pooled in 500 μL TRIzol. There were two biological replicates of 4 fish each for each sampling point, treatment and temperature. Total RNA isolation was conducted both for larvae and adults following TRIzol manufacturer's specifications in combination with the RNeasy mini kit (Quiagen) for RNA purification after DNaseI treatment. One microgram of total RNA was always used to obtain cDNA by the SuperScript III first-strand synthesis supermix for qRT-PCR (Invitrogen).

2.4 Gene expression

A group of known proinflammatory and antiviral genes (IL-1β, iNOS, TNF-α, TLR3, IFN-I, IFNγ, IRF3, MDA-5 and Mx) was selected in order to quantify their expression pattern by real time PCR (qPCR) both under naïve conditions and after poly I:C stimulation or VHSV infection. Amplification was carried out using specific primers (Table 1), which were designed according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). Primers efficiency was then validated based on the slope of a standard curve line and a melting curve analysis was performed to verify that no primer dimers were amplified. One microliter of 10-fold diluted cDNA template was mixed with 0.5 µl of each primer (10 µM) and 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. All reactions were carried out as technical triplicates. The comparative C_t method ($\Delta\Delta C_t$ method) was used to determine the expression level of analysed genes [37]. The expression of the selected genes was normalised using the 18S rRNA as a housekeeping gene (18S-F: ACC ACC CAC AGA ATC GAG AAA and 18S-R: GCC TGC GGC TTA ATT TGA CT), as 18S rRNA was constitutively expressed independently of the temperature and the stimulation/infection. Fold change units were calculated by dividing the normalised expression values of stimulated/infected tissues by the normalised expression values of the controls. For the biological replicates the average relative level of expression from each replicate was considered as a single point and the mean and standard error calculated.

2.5 Statistical analysis

Data were compared using the Student's t-test and Analysis of Variance (ANOVA) to evaluate gene expression differences and the effect of the temperature. In the case of the

effect of the temperature, all the sampling points and the two biological replicates were considered together for ANOVA analysis in larvae, giving a matrix of 40 values. In adults, for temperature comparison 2x2 (t test) the number of points considered was 12 in each comparison.

3. RESULTS

3.1 Constitutive gene expression during early zebrafish development

The expression of the selected proinflammatory and antiviral genes (IL-1β, iNOS, TNF-α, TLR3, IFN-I, IFNγ, IRF3, MDA-5 and Mx) during the zebrafish early development was measured by qPCR using the control larvae for each temperature. It should be taken into account that 28 ºC is the normal temperature for zebrafish development, while 15 ºC experiment only reflects the acute temperature change and a general variation in rate of metabolism rather than the stage of development for the controls. At 15 ºC most of the genes analysed showed no amplification at 2 dpf, which was especially true for IL-1β, iNOS, TNF-α and IFN-I. Three days later (5 dpf), the expression values of most of these genes were still low, only reaching positive reaction thresholds ($28 \leq C_t \leq 32$) with moderate amounts of target nucleic acid at 8 dpf. Also, most of the genes analysed exhibited a profile with two maximum peaks at 15 ºC, with the exception of TLR3 and IRF3 (Figure 1). The first peak was registered at the 8-17 dpf window and the second one at the 20-26 dpf window. The expression profile of TLR3 clearly increased during zebrafish development while that of IRF3 seemed not to be regulated.

At 28 ºC the gene expression profiles were quite different. The expression values at 2 dpf showed no amplification for most of the genes. However, the positive reaction threshold was reached for all of the genes at 5 dpf, with the exception of IFNγ and Mx.

In general, all of the genes presented a unimodal profile with low expression values from 2 dpf up to 14-17 dpf, with a maximum expression peak around 20 dpf that decreased again from 23 dpf up to 29 dpf (Figure 2). Taking into account both temperatures, the expression values between the two biological replicates were more similar at 28 ºC than at 15 ºC, which seems to indicate a high response variability under condition where the normal developmental pathway is disturbed. As expected, the expression values were generally higher at 28 ºC as compared to the values observed at 15 ºC. IL-1β, iNOS, TNF-α and TLR3 were the most highly expressed genes at 15 ºC, whereas the rest of the genes were poorly expressed. At 28 ºC, iNOS was more highly expressed, while TNF-α, TLR3 and IRF3 displayed the lowest expression values (Figures 1 and 2).

3.2 Gene expression after poly I:C stimulation or VHSV infection during early zebrafish development

The modulation of the gene expression after stimulation with poly I:C was also studied. Moreover, in order to determine the effect of low temperature on the response to a live pathogen, VHSV infections were conducted at 15 ºC. Taking into account a two-fold increase in the significance level, no changes between control and stimulated/infected fish were observed for most of the genes at 15 ºC, particularly TNF-α, TLR3, IFNγ and MDA-5. IRF3 and Mx responded significantly to poly I:C stimulation starting at 11 dpf and 5 dpf, respectively. IL-1β and iNOS were upregulated by > 2-fold at 11-17 dpf and 23-29 dpf, coinciding with higher expression values for the controls. In the case of IFN-I the expression values observed at 2 dpf should be considered cautiously as no amplification was observed for the control group. This gene presented a >2-fold increase at 5 dpf and at the 20-26 dpf window upon poly I:C

stimulation. Also, it was generally observed that poly I:C stimulation led to higher fold increases in gene expression as compared to the expression levels observed during VHSV infection (Figure 3).

At 28 ºC, all of the genes analysed showed significant differences in the expression levels between control and stimulated fish, in at least some of the sampling points (Figure 4). Transcript levels for TLR3 and TNF-α were slightly modulated at 28 ºC as occurred at 15 ºC . However the situation changed for IFNγ and MDA-5, which exhibited fold increase values > 2 at practically all of the sampling points. The fold change for IRF3 was > 2 for most of the sampling points, regardless of the temperature, but exhibited higher expression at 28 ºC. IFN-I displayed expression differences > 2 in the first stages of zebrafish development, but was < 2 from 20 dpf to 29 dpf, which contrasted with observations made at 15 ºC. IL-1β and iNOS presented a more random behaviour, as was observed at 15 ºC, but resembled the pattern of IFN-I expression. Between control and stimulated fish, Mx was the gene in which differences were most evident, showing the highest fold increase value (549.23 maximum) among all of the genes analysed (Figure 4). Also, the differences between both temperatures were more obvious for Mx. For instance, a maximum 549.23-fold increase was observed at 11 dpf (28 ºC) as compared to a 12.13-fold increase at 15 ºC for the same sampling point (Figure 5). The results at 28 ºC showed a general trend of more robust responses during the first stages of the development (5-17 dpf) that decreased during the second half of the month (Figure 4).

3.3 Gene expression after poly I:C stimulation in adult zebrafish

Based on the results observed in larvae, the expression of Mx was assessed in adult zebrafish, at both 15 and 28 ºC and in a group of fish acclimated to 15 ºC for 1

week prior to the stimulation. In adults, the analysis of Mx expression was conducted at 3, 6 and 24 hps (Figure 6). T tests were performed to assess differences between each temperature for each sample point. Significant differences were obtained at 6 hps for all temperature combinations: 15 ºC *versus* 15 ºC acclimation ($p<0.05$); 15 ºC *versus* 28 ºC ($p<0.01$); 15 ºC acclimation *versus* 28 ºC ($p<0.01$). In addition, significant differences were observed at 24 hps for 15 ºC *versus* 28 ºC and 15 ºC acclimation *versus* 28 ºC ($p<0.05$). The results showed significant expression values (fold change values > 2) at 3 hps for 15 ºC and at 6 hps for both 15 ºC and 28 ºC. After 24 hps, Mx expression was significantly different from controls at all temperatures. Contrary to what happens in larvae, there were not important differences on Mx expression after 24 hps at any of the three situations analysed (15 ºC, 15 ºC with acclimation and 28 ºC). Moreover, the expression values never reached the levels displayed in larvae in the first 17 days of development (Figure 4).

4. DISCUSSION

Zebrafish are currently being used as a model to study many biological processes across many fields, including human biomedicine. The zebrafish provides, among others advantages, a relatively simple tool for genetic manipulation and a fast development. It is well known that temperature influences biological processes. This is particularly evident for fish immune responses [38], in which the adaptive immune response is relatively inefficient, given their evolutionary status and poikilothermic nature. Thus, innate immune response is considered instant and relatively temperature independent as compared to the acquired immune response [39-41].

During ontogeny, (only considering 28 ºC as the normal developmental temperature for zebrafish), our results show that the majority of the genes analysed

reached a positive reaction threshold as early as 5 dpf. This is not surprising as oviparity necessitates a rapid development of the immune system. It was also noted that higher expression values were observed for most of the genes at all the sampling points at 28 ºC as compared to 15 ºC. This immune response plasticity regarding the temperature was already reported in amphibians over the course of a year in temperate ecosystems. During long-term periods of low temperature, some immune system parameters decline to a lower level. This is thought to be due to a trade-off between temperature-dependent proliferation rates of typical pathogens and the cost of immunity [42,43]. The antiviral activity-related genes tested here (IFN-I, IFNγ, IRF3, MDA-5 and Mx) were almost completely inhibited at 15 ºC. However, in the case of IL-1β, iNOS and TNF-α the expression differences between both temperatures were not so obvious. As proinflammatory cytokines with pleiotropic effects, their role could be more important during conditions of stress. Also, it has been reported in humans that stress increases the plasma levels of TNF-α [8]. TLR3 displayed high expression values at 15 ºC, which may be related to its important role as an immune receptor, but exhibited low expression values at 28 ºC, although we do not have a clear explanation for that.

A progressive increase in the expression of the analysed cytokines during development was generally observed at 28 ºC. Around 23 dpf the expression tended to decrease, which correlates with the time that the adaptive immune system is supposed to be fully active in this species [33]. It is possible that the early expression of the cytokines examined, and possibly several others, is involved in the maturation of the adaptive immune system. Interestingly, IFNγ displayed positive C_t values around 17 dpf at 28 ºC, although it was already detected at 8 dpf (C_t= 34). This expression pattern for IFNγ seems to be in agreement with its biological activity, as it is mainly a Th1 cytokine secreted by T lymphocytes and NK cells under specific activation conditions

[44-46]. This is the first time that IFNγ expression was detected as early as 8 dpf during development, highlighting that lymphocyte activity can occur before the adaptive immune system is completely mature (4-6 wpf) [33].

Regarding gene modulation in larvae after poly I:C stimulation or VHSV infection, the results showed no significant differences (fold change < 2) between infected and control fish for TNF-α, TLR3, IFNγ and MDA-5 for all sampling points at 15 ºC. Similar results were obtained for TLR3 and other viral recognition-related proteins (TLR22, MAVS and RIG-1) after zebrafish SVCV challenge [47], demonstrating limited antiviral activity. In contrast, IRF3 and Mx, which are an interferon I regulator and one of the best known interferon effector genes, respectively, presented significant response levels from 11 dpf (IRF3) and 5 dpf (Mx) after poly I:C stimulation, although the expression magnitude for Mx was higher (maximum of 12.13-fold increase value at 11 dpf). The interferon system is the main vertebrate (including fish) mechanism to fight viral infections [48-54]. Therefore, it was not surprising to observe fold increase values > 2 for these two genes following stimulation from early stages after hatching. Several studies in fish and other vertebrates have also reported a significant upregulation of IRF3 in a variety of tissues and cell culture systems after poly I:C stimulation or cold acclimation [53,55-57]. The upregulation of Mx and other interferon-induced genes was already observed in previous work, both after transcriptome analysis of turbot nodavirus infection and poly I:C stimulation [57] and after nodavirus infection in sea bream and sea bass [58]. Furthermore, Plant et al. [59] and Fernandez-Trujillo et al. [60] had also observed a more rapid and higher expression level of Mx after poly I:C induction.

At 28 ºC the situation was completely different, as all of the genes (above all the antiviral genes) presented expression differences as compared to the control after poly

I:C stimulation in at least some of the sampling points. It was especially remarkable that the expression values for the Mx gene was so high, with a 549.23-fold increase at 11 dpf compared to a 12.13-fold increase for the same sampling point at 15 ºC. In our opinion, this overexpression of Mx suggests a high competence of zebrafish larvae against viral infections. The results also showed as well a general profile with a higher response in the first 17 days of the development that then decreased during the following days. According to Lam et al. [33], the adaptive immune system in zebrafish is considered functional and morphologically mature at 4-6 wpf. The major immune events leading to immunocompetence take place between 2-4 wpf, which coincides with the larvae to juvenile transitory phase (from 14 to 28-42 dpf proposed by Brown [61]). This synchronised transition with the maturation of the immune system likely corresponds to a developmental strategy balancing food availability, metabolic fitness, hormonal factors and the developing immune system [33]. In any case, during that 3-week window, larvae cannot mount an adaptive immune response to potential pathogens and presumably must depend solely on innate responses. This could be the reason why the innate immune response seemed to be overshadowed by the rudiment adaptive immune system during the second half of the development in our study.

In general, after conducting statistical analyses to determine the effect of the temperature in the expression of all of these genes after poly I:C stimulation, we can conclude that no significant differences were obtained for inflammation-related genes (IL-1β and iNOS) with the exception of TNF-α ($p<0.001$). However, significant differences ($p<0.0001$) were displayed for all of the antiviral genes. It seems that those genes more directly related to the viral infection, independent of their regulation following stimulation, were very sensitive to low temperatures. Nevertheless, those genes involved in generalised immune responses were much less affected at 15 ºC.

Given the results obtained in larvae for the Mx gene, its expression was assessed in adults following poly I:C stimulation. VHSV infection was not assessed herein, as a similar VHSV infection study was already conducted in previous work and did not exhibit any important modulation of Mx following infection [62]. There were no significant differences in the response of the fish with or without 15 ºC acclimation and, although the results showed significant differences at 6 hps (for all temperature combinations) and at 24 hps (between low and high temperatures), the Mx expression differences were visibly reduced at the end of the experiment (24 hps). In fact, Mx expression of fish that had been acclimated before stimulation was even higher as compared to 28 ºC. Comparison between larvae and adult experiments was not strictly possible due to different administration routes of poly I:C. However, this comparison provided some clues regarding the differences in innate immune response between larvae and adults. The results indicate that Mx expression differences were greater between 15 and 28 ºC in larvae than in adults, which suggests a major susceptibility to the temperature, in regards to the Mx response, in larvae. This is especially true for the first 17 days of development, when zebrafish larvae depend completely on the innate immune system to fight against non-self particles. On the other hand, the fold increase values in adults were lower (52.58-fold change value as maximum) than that observed in larvae (549.23 as maximum). This could explain why larvae seem to be more resistant to viral infection when compared to adult zebrafish, as we have observed during various experimental infections (data not shown).

5. CONCLUSIONS

From the results obtained in this study, we could conclude that there was an early expression of the cytokines analysed (including IFNγ), even at temperatures below

that found during normal development (15 ºC *versus* 28 ºC). Temperatures lower than 28 ºC dramatically modified the expression profiles of several genes in larvae after stimulation or infection. On the contrary, in adults, the differences between temperatures regarding Mx expression were much lower. Taken together, the results of Mx expression in adults and larvae seem to suggest that Mx plays a crucial role during viral infections in larvae, when the adaptive immune response is not completely developed. Mx was also upregulated in adult zebrafish, which suggests the important role that this gene may play against viruses even when the adaptive response is mature and functional.

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FIGURE LEGENDS

Figure 1. mRNA levels of proinflammatory and antiviral genes during early development of larvae zebrafish maintained 24 hours at 15 ºC. All qPCR reactions were carried out as technical triplicates and the expression level of analysed genes was normalised to the 18S rRNA. Expression units were calculated by dividing the normalised expression values of each sample point by the normalised expression value at 8 dpf. Each bar represents the mean and standard error of two biological replicates. Numbers on the abscise axis indicate days post-fertilization.

Figure 2. mRNA levels of proinflammatory and antiviral genes during early development of larvae zebrafish maintained at 28 ºC. All qPCR reactions were carried out as technical triplicates and the expression level of analysed genes was normalised to ribosomal 18S rRNA. Expression units were calculated by dividing the normalised expression values of each sample point by the normalised expression value at 8 dpf. Each bar represents the mean and standard error of two biological replicates. Numbers on the abscise axis indicate days post-fertilization (dpf).

Figure 3. Expression level of proinflammatory and antiviral genes during early development of larvae zebrafish after poly I:C and VHSV challenge at 15 ºC. All qPCR reactions were carried out as technical triplicates and the expression level of analysed genes was normalised using the 18S rRNA. Fold change units were then calculated by dividing the normalised expression values of stimulated/infected tissues by the normalised expression values of the controls. Each bar represents the mean and standard error of two biological replicates.

Figure 4. Expression level of proinflammatory and antiviral genes during early development of larvae zebrafish after poly I:C challenge at 28 ºC. All qPCR reactions were carried out as technical triplicates and the expression level of analysed genes was normalised to 18S rRNA. Fold change units were then calculated by dividing the normalised expression values of stimulated tissues by the normalised expression values of the controls. Each bar represents the mean and standard error of two biological replicates. *, this sampling point was eliminated to avoid artifacts due to the very low expression level observed in controls at this sampling point.

Figure 5. Comparison of the expression levels of the analysed genes during early development of larvae zebrafish after poly I:C challenge at 15 and 28 ºC. All qPCR reactions were carried out as technical triplicates and the expression level of analysed genes was normalised to 18S rRNA. Fold change units were then calculated by dividing the normalised expression values of stimulated tissues by the normalised expression values of the controls. Each bar represents the mean and standard error of two biological replicates. *, this sampling point was eliminated to avoid artifacts due to the very low expression level observed in controls at this sampling point.

Figure 6. Mx expression level of adult zebrafish after poly I:C challenge at 15 ºC and 28 ºC and for fish previously acclimated to 15 ºC before challenge. All qPCR reactions were carried out as technical triplicates and the expression level of analysed genes was normalised to 18S rRNA. Fold change units were then calculated by dividing the normalised expression values of stimulated tissues by the normalised expression values of the controls. Each point represents the mean and standard error of two biological

replicates. 15 ºC A, fish acclimated to 15 ºC for 1 week prior to poly I:C challenge. Numbers on the abscise axis indicate hours post-stimulation.

Table 1. Primer sequences designed for qPCR analysis of selected genes. 18S rRNA was chosen as housekeeping gene.

Primer	**Sequence**
F-IL-1β	TTCCCCAAGTGCTGCTTATT
R-IL-1β	AAGTTAAAACCGCTGTGGTCA
F-iNOS	GGA GAT GCA AGG TCA GCT TC
R-iNOS	GGC AAA GCT CAG TGA CTT CC
F-TLR3	AAGCCCATCATGCTCTTCAT
R-TLR3	AAGGCCAGTAGAGGACACATTT
F-TNF-α	ACCAGGCCTTTTCTTCAGGT
R-TNF-α	GCATGGCTCATAAGCACTTGTT
F-INF-I	GTCTACTTGCGAATGGCTTG
R-INF-I	GGTCCTCCACCTTTGACTTG
F-INFγ	GACGTATGCAGAAACGCTATGG
R-INFγ	ATGCTTTAGCCTGCCGTCTCT
F-IRF3	CTGTACCCAGTTCAGCATTCC
R-IRF3	GGAAAGTTTCTCTTCCACACAGA
F-MDA-5	GAATCAGAATGTTCGCGTGTGT
R-MDA-5	CCTCGTCAGGGCTAGATTTGG
F-Mx	CGCTGTCAGGAGTTCCGTTAC
R-Mx	TTCCGCTGGGTCATCAAAGT
F-rRNA18S	ACCACCCACAGAATCGAGAAA
R-rRNA18S	GCCTGCGGCTTAATTTGACT

Figure 1.

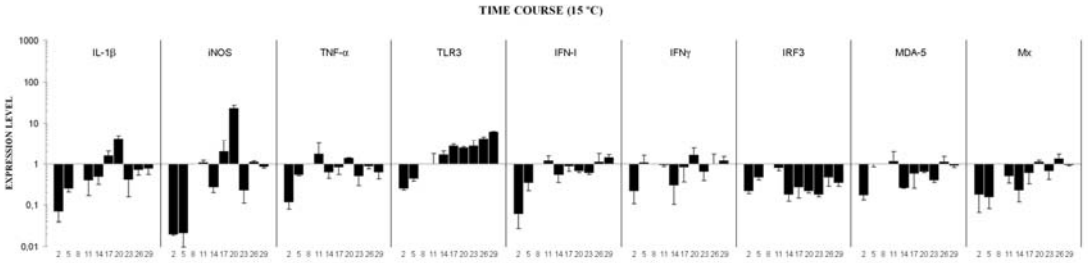
TIME COURSE (15 °C)
EXPRESSION LEVEL
1000
100
10
1
0,1
0,01
IL-1β
iNOS
TNF-α
TLR3
IFN-I
IFNγ
IRF3
MDA-5
Mx
2 5 8 11 14 17 20 23 26 29

Figure 2.

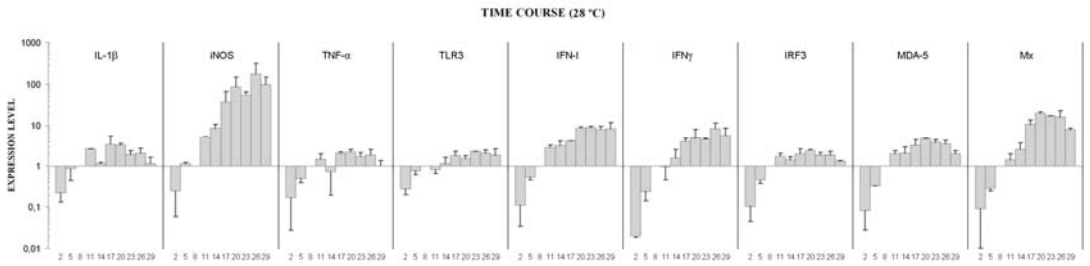
TIME COURSE (28 °C)
EXPRESSION LEVEL
1000
100
10
1
0,1
0,01
IL-1β
iNOS
TNF-α
TLR3
IFN-I
IFNγ
IRF3
MDA-5
Mx
2 5 8 11 14 17 20 23 26 29

Figure 3.

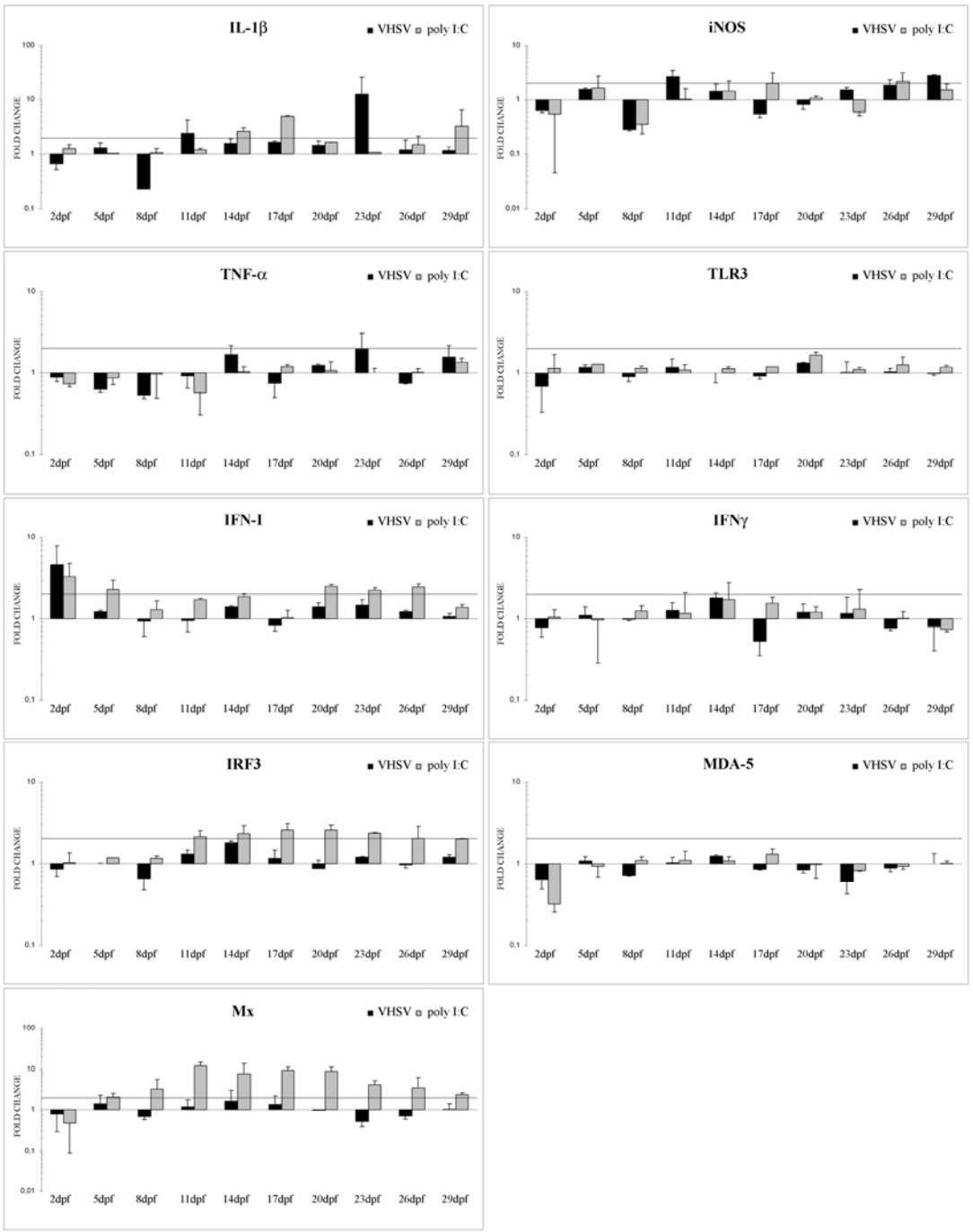
IL-1β
iNOS
TNF-α
TLR3
IFN-1
IFNγ
IRF3
MDA-5
Mx
VHSV
poly I:C
FOLD CHANGE
2dpf
5dpf
8dpf
11dpf
14dpf
17dpf
20dpf
23dpf
26dpf
29dpf

Figure 4.

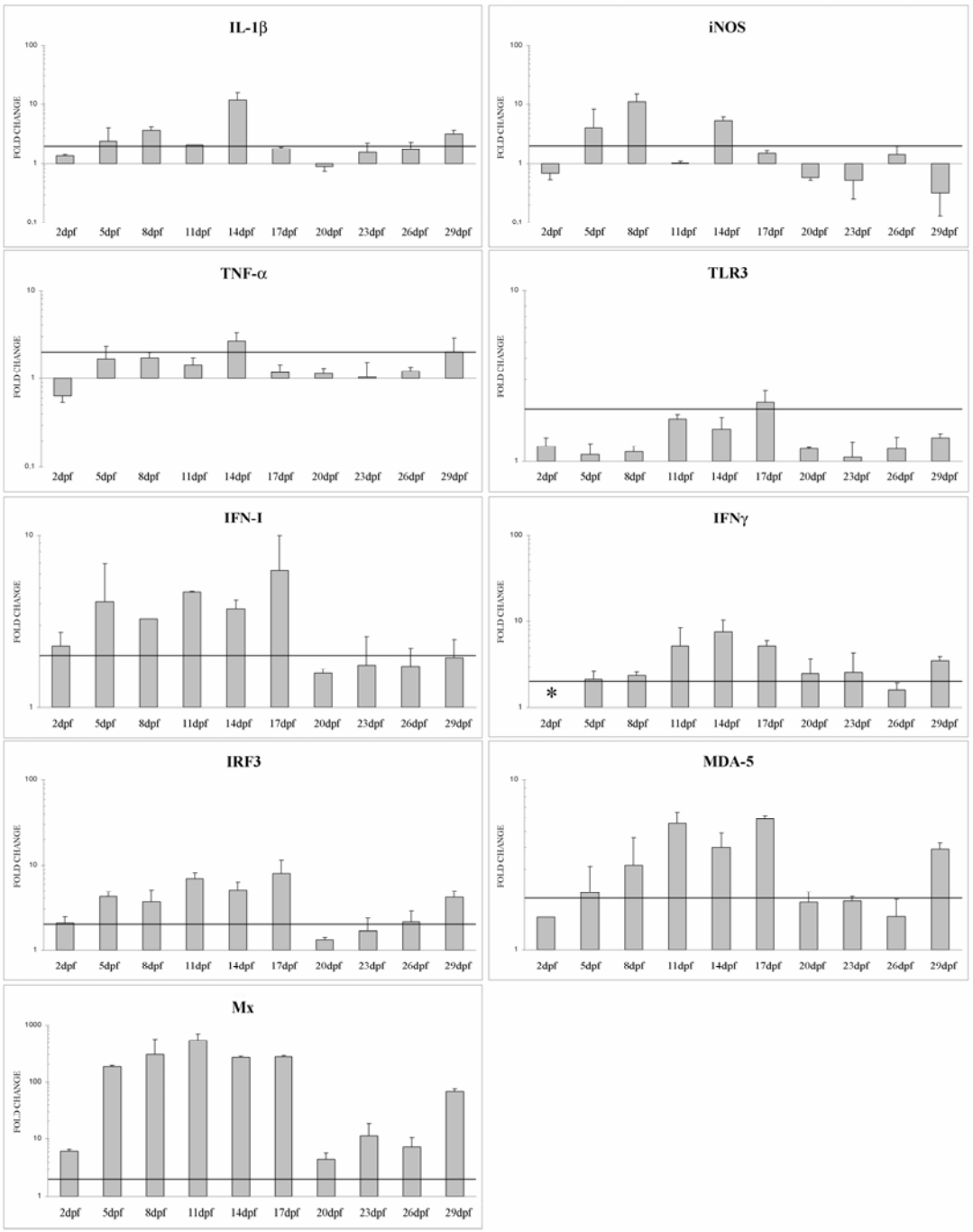
IL-1β
iNOS
TNF-α
TLR3
IFN-I
IFNγ
IRF3
MDA-5
Mx
FOLD CHANGE
2dpf
5dpf
8dpf
11dpf
14dpf
17dpf
20dpf
23dpf
26dpf
29dpf
*

Figure 5.

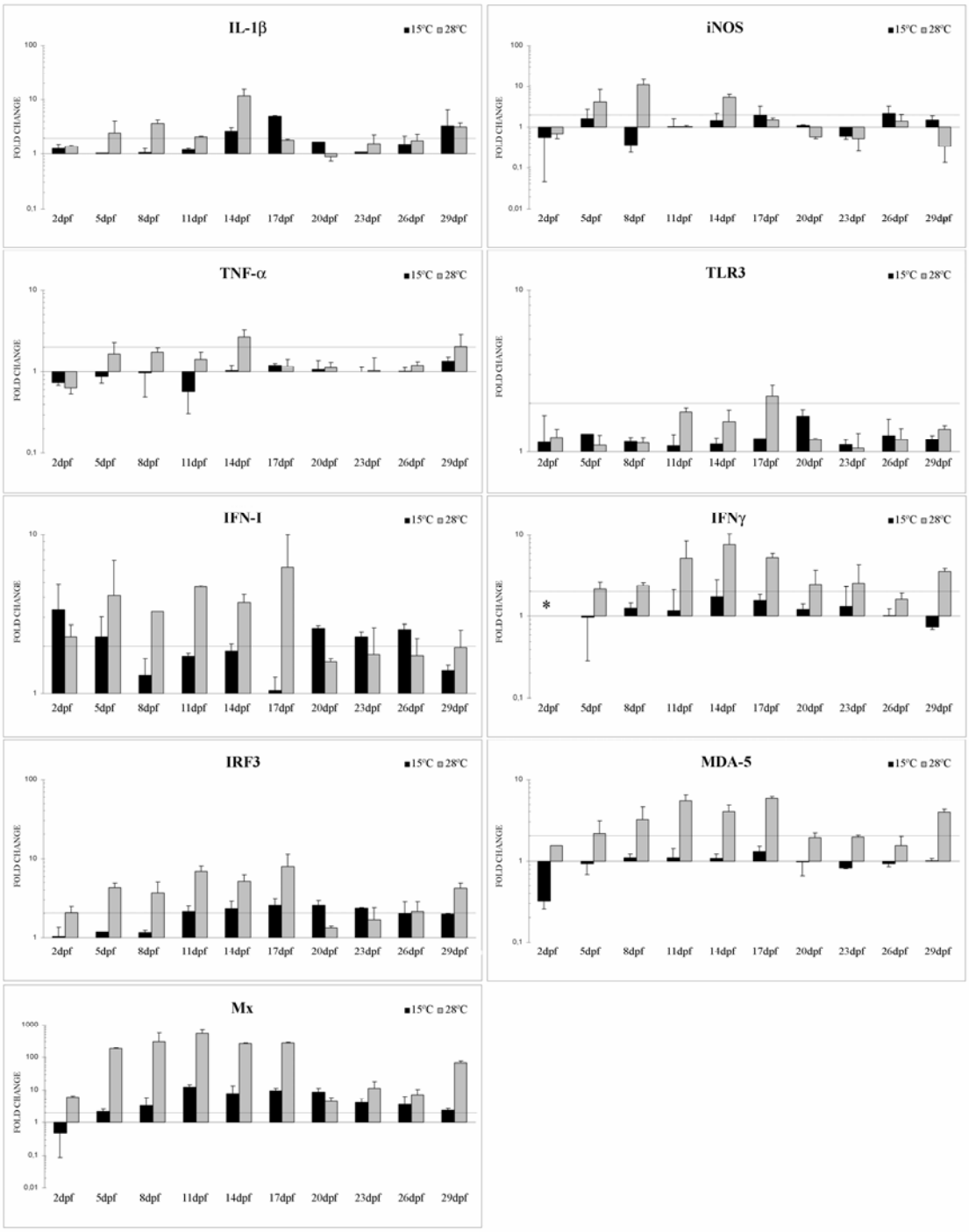
IL-1β
iNOS
TNF-α
TLR3
IFN-1
IFNγ
IRF3
MDA-5
Mx
15°C 28°C
FOLD CHANGE
2dpf 5dpf 8dpf 11dpf 14dpf 17dpf 20dpf 23dpf 26dpf 29dpf
*

Figure 6.