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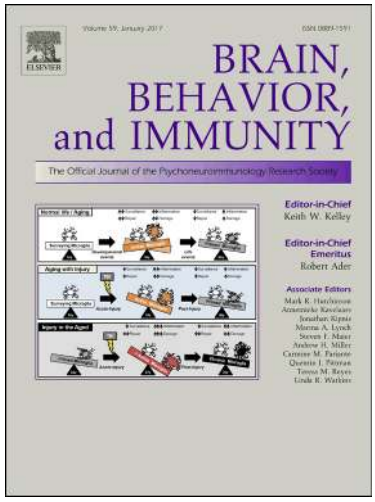

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The expression of TRPV channels, prostaglandin E2 and pro-inflammatory cytokines during behavioural fever in fish

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Abstract

A fever, or increased body temperature, is a symptom of inflammation, which is a complex defence reaction of the organism to pathogenic infections. After pathogens enter the body, immune cells secrete a number of agents, the functions of which stimulate the body to develop a functional immune and fever response. In mammals it is known that PGE_2 is the principal mediator of fever. The extent to which PGE_2 and other pro-inflammatory cytokines such as TNF-α, IL-6, or IL-1β could be involved in the induction of behavioural fever in fish remains to be clarified. Several members of the transient receptor potential (TRP) family of ion channels have been implicated as transducers of thermal stimuli, including TRPV1 and TRPV2, which are activated by heat. Here we show that members of the TRP family, TRPV1 and TRPV4, may participate in the coordination of temperature sensing during the behavioural fever. To examine the behavioral fever mechanism in *Salmo salar* an infection with IPNV, infectious pancreatic necrosis virus, was carried out by an immersion challenge with 10×10^5 PFU/mL^{-1} of IPNV. Behavioural fever impacted upon the expression levels of both TRPV1 and TRPV4 mRNAs after the viral challenge and revealed a juxtaposed regulation of TRPV channels. Our results suggest that an increase in the mRNA abundance of TRPV1 is tightly correlated with a significant elevation in the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and PGE_2) in the Pre-Optic Area (POA) and cytokine release in plasma. Together, these data indicate that the reduction of TRPV4 expression during behavioural fever may contribute to the onset of behavioural fever influencing movement toward higher water temperatures. Our data also suggest an effect of TRPV channels in the regulation of behavioural fever through activation of EP3 receptors in the central nervous system by PGE_2 induced by plasma-borne cytokines. These results highlight for first time in mobile ectotherms the key role of pro-inflammatory cytokines and TRPV channels in behavioural fever that likely involves a complex integration of prostaglandin induction, cytokine recognition and temperature sensing.

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Keywords

Ectotherm, behavioural fever, cytokine, TRP channels, virus

Abbreviations

IPNV, Infectious Pancreatic Necrosis Virus; IL-1β interleukin-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; COX-2 cyclooxygenase-2; PGE_2, prostaglandin E2; TRPV1, transient receptor potential cation channel, subfamily V, member 1; TRPV4, transient receptor potential cation channel, subfamily V, member 4, POA; Hypothalamic Preoptic Area, GABA; aminobutyric acid.

1. Introduction

Most animals increase their body temperature above its normal thermal set point in response to infection, a phenomenon known as fever (Evans et al., 2015). The fever mechanism is initiated after pathogen recognition and the consequent induction of specific signalling pathways (Bicego et al., 2007; IUPS, 2001; Rakus, Ronsmans, & Vanderplasschen, 2017). Briefly, individual recognise pathogens via germline encoded pathogen pattern recognition receptors (PRRs) that sense particular structures of the microorganisms (pathogen-associated molecular patterns, PAMPs), initiating a well-orchestrated defence response (Boltaña et al., 2011). The defence signalling pathway involves different cellular interactions (macrophages, neutrophils, leukocytes, etc) that are initially activated through PAMP-PRR interaction followed by an inflammatory response. Consequently, the recruitment of leukocytes and the activation of antimicrobial effector systems promote the release of endogenous signals such as pyrogenic cytokines that finally drive an increase of body temperature through central nervous system activation (Atkins & Bodel, 1979; Blatteis, 2006; Dinarello, 2004; Dinarello et al., 1986;

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Medzhitov, 2007; Mogensen, 2009; Netea et al., 2000). Locally produced cytokines migrate through the bloodstream to the hypothalamic Preoptic Area (POA) that is involved in the development and modulation of the fever mechanism (Banks, 2005; Boulant, 2010; McKinley et al., 2003). Once in the POA, pyrogenic cytokines activate transcription factors including nuclear factor-kappa-β (NF-kβ) and the activator of transcription-3 (STAT3) (Laflamme & Rivest, 1999; Rummel et al., 2005). These transcription factors interact with the key genes involved in the biosynthesis of prostaglandin E_2 (PGE_2), corresponding to those codifying for cyclooxygenase 2 (COX-2) and microsomal PGE synthase 1 (mPGES-1) (Nadjar et al., 2005; Rummel et al., 2011; Rummel et al., 2006; Saha et al., 2005)

PGE_2 is considered as the main pyrogenic mediator of fever in mammals (Engström et al., 2012; Matsumura et al., 1998; Yamagata et al., 2001). It has been reported that PGE_2 can be synthetized directly in the brain or secreted by blood or tissue macrophages. In the brain, it binds to PGE_2 receptors of GABAergic neurons of the Median Preoptic Nucleous (MnPO) of the POA (Rakus, Ronsmans, & Vanderplasschen, 2017). PGE_2 receptors have been classified in four subtypes: EP1 – EP4. Among them, EP3 has been demonstrated to have a critical role in the induction of fever (Coleman et al., 1994; Lazarus et al., 2007; Ushikubi et al., 1995). PGE_2 binds the EP3 receptor blocking the activity of neurons of the Dorsomedial Hypothalamic Nucleous (DMH) and the medullar rostral raphe (rMR). EP3 receptor blockade drives intrinsic thermogenesis in the adipose tissue, muscle shivering and peripheral vasoconstriction, preventing heat loss (Nakamura, 2011; Nakamura et al., 2002; Saper et al., 2012).

In contrast, the inhibition of heat loss by mechanisms such as shivering or peripheral vasoconstriction have not been reported in most ectothermic animals, which have very limited ability to manipulate their body temperature through physiological readjustments (Stahlschmidt & Adamo, 2013). Instead, temperature regulation is highly influenced by the temperature of the surrounding environment. Throughout evolution, ectotherms have developed specific adaptive traits in order to increase the efficacy of their metabolic machinery and overall regulatory

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responses including immunity, growth, and energetics. Amongst these thermoregulatory behaviours driving optimal temperature selection is an important adaptive trait (Final Preferendum Temperature or FPT) responding to specific needs (Anderson et al., 2013; Elliot et al., 2002; Golovanov, 2006; Stahlschmidt & Adamo, 2013). It has been reported that ectotherms migrate to warmer zones in order to increase their body temperature above FPT after a pathogen infection, stress or exogenous pyrogen injection. This behaviour is known as behavioural fever and is part of a defence system that can increase the survival in infected individuals (Boltaña et al., 2013; Evans et al., 2015; Kluger, 1979; Rey et al., 2015; Reynolds & Casterlin, 1982). However, the underpinning mechanisms by which individuals perceive and integrate temperature changes during the behavioural fever have been poorly studied.

In ectotherms, temperature detection is an important preventive system against potential physiological damage resulting from inappropriate temperature driven allostatic loading (Boltaña et al., 2017). Fish subjected to a simple experimental manipulation actively regulate physiological responses by shifting positions within a thermal gradient, thus highlighting a "*fine*" control of regulatory responses (Boltaña et al., 2013; Boltaña et al., 2017; Rey et al., 2017). Thermal choice, and the associated responses, are elicited by thermal perception through the transient receptor potential (TRP) channel family. TRP channels belong to a large family of thermal receptors directly activated by hot or cold temperature ranges and are extremely sensitive temperature variation (Feng, 2014; Song et al., 2016). These sensors orchestrate the homeostatic repertoire by which individuals modify their thermal behaviour that in turn results in a suitable regulatory response (Morrison & Nakamura, 2011).

The regulation of behavioural fever in ectotherms is evolutionarily related to fever induced mechanisms found in endotherms (Evans et al., 2015; Rakus, Ronsmans, & Vanderplasschen, 2017), including the role of exogenous pyrogens as inductors (e.g. Boltaña et al., 2013; Roth & Blatteis, 2014; Sherman et al., 1991; Soares et al., 2009), or the role of prostaglandins as mediators of the fever response (e.g. Boltaña et al., 2013; Myhre et al., 1997). Collectively,

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published data suggests that cytokines such as interleukin 1β (IL-1β), IL-6, tumor necrosis factor α (TNF-α) and interferons act as endogenous pyrogens (Dinarello, 1999; Netea et al., 2000). In ectotherms, TNF-α and PGE_2 have been demonstrated to act as mediators for behavioural fever (Boltaña et al., 2013; Rakus, Ronsmans, Forlenza, et al., 2017). To date the functional mechanisms by which ectotherms regulate their thermoregulatory behaviour during an infection, remain poorly understood (Rakus, Ronsmans, & Vanderplasschen, 2017). For fish, we propose that changes in thermoregulatory behaviour driven centrally by TRPs act in concert with inflammatory cytokine signalling to develop and modulate the behavioural fever response. This study provides fundamental insights into the molecular mechanisms that drive the activation of behavioural fever in ectotherms. To test this, we have used an Atlantic salmon (*Salmo salar*) model to ask the following questions:

(1) Does a viral infection cause behavioural fever in *Salmo salar*?

If so:

(2) Is there a homology between regulatory pathways of fever in endotherms and behavioural fever in ectotherms regarding the role of endogenous pyrogens?

(3) Are TRPV channels involved in the regulation of behavioural fever in fish?

2. Materials and Methods

2.1. Fish husbandry and experimental conditions

All *S. salar* thermal experiments were carried out at the ThermoFish Lab, Biotechnology Centre, University of Concepción, Concepción, Chile. Fish were handled in accordance with the "International Guiding Principles for Biomedical Research Involving Animals" established by the European Union Council (2010/63/EU). 3,000 *S. salar* were obtained in December 2016 from AquaGen S.A., Melipeuco, Chile, and were maintained in two recirculating freshwater systems (210 × 150 × 90 cm). Each system had a flow rate of 5 m^3 h^{-1}, and water was U.V.-sterilized. Each system contained three independent tanks (60 × 140 × 70 cm). Water temperature was measured

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twice per day (7 ± 0.7 °C). Dissolved oxygen was also measured daily and always remained above 9 mg L^{-1}. Ammonia, nitrite, and pH were measured twice per week. Total ammonia and nitrite concentrations in each tank were maintained under 0.05 and 0.01 mg L^{-1}, and pH remained at 8.0 ± 0.5. A 24-hr dark cycle photoperiod was used until the viral challenge.

2.2. Infectious pancreatic necrosis virus (IPNV) challenge

2.2.1 Virus production.

Chilean isolates of Infectious Pancreatic Necrosis virus (IPNV) were supplied as a frozen suspension of salmon head kidney tissue homogenate that had been diluted 1:10 in phosphate-buffered saline pH 7.2 (PBS) and centrifuged. We used the infectious supernatant and embryo cells (CHSE-214) of chinook salmon *Oncorhynchus tshawytscha* for virus isolation, plaque cloning, and subsequent passage by sequential transfer in cell culture. Monolayer cultures of CHSE-214 cells were maintained in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum. For virus production, drained monolayer cultures were infected at a level of infection (multiplicity of infection) of 0.01 plaque-forming units (PFU) per cell. Virus was allowed to adsorb for 1 h at 15 °C and the cells were overlaid with EMEM containing 5% fetal bovine serum.

2.2.2 Viral Challenge

In vivo infection of salmon with IPNV was carried out by immersion using dechlorinated water from stock tanks following protocols previously described (Taksdal et al., 1997). Clarified supernatant from IPNV-infected CHSE-214 cell monolayers (10 x 10^5 PFU/mL^{-1}) was added to water in 5 L water tanks containing the fish (n = 20). In parallel, fishes in a control tank were similarly treated by adding 100 ml virus free cell culture supernatant to the water. After 120 minutes, fish were separated in two groups (n = 10 each) and then placed in the experimental thermal gradient tanks (see 2.3), either under restricted thermal range (15±0.41 ºC, RTR) or at

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15±7.4 ºC (wide thermal gradient, WTR). Experimental groups were: (i) control (Mock, no infected, n=10 fish by replicate), (ii) immersion challenge with IPNV in a wide thermal gradient tank (WTR-IPNV, 15±7.4 ºC, n=10 fish by replicate), and (iii) immersion challenge with IPNV restricted thermal gradient tank (RTR-IPNV, 15±0.41 ºC, n=10 fish by replicate). Abdominal distension, exophthalmia, impaired swimming and skin/fin base haemorrhages was recorded for each fish. In the thermal gradient, behavioural data was recorded as described below throughout the challenge experiments (see 2.3). Three independent replicates were carried out (N=90 fish) (Fig. 1).

2.2.3. Infectious pancreatic necrosis virus recovery (qPCR)

Quantitative PCR (qPCR) was used for IPNV load estimation by targeting the virus segment VP2 region using primer WB117 and Universal ProbeLibrary probes (UPL) (Supplementary Table 1) as previously described Avendaño-Herrera et al. (2017), Calleja et al. (2012) and Ørpetveit et al. (2012). Total, RNA was extracted from head kidney and spleen following the protocol described in section 2.7

2.3. Behavioural studies

The experimental thermal gradient was carried out in 2.5 m^3 tanks (105 x 15 x 15 cm) divided with five transparent Plexiglas screens to create six equal interconnected chambers. Each screen had a hole at the centre (3 cm diameter; 10 cm from the bottom) to allow fish to move freely between chambers. Three video cameras provided continuous monitoring of each tank chamber. During the experiment, temperatures were recorded for 10 s every 15 min throughout daylight hours (24 h = 96 recorded events). Four groups of fish (n = 10 for each group) were introduced into chamber 4 in the evening and filming began at 6:00 the next day, providing a 12-h acclimation period. The distribution of fish into the six compartments was monitored over time with video cameras and the number of fish in each compartment was counted manually from the

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images captured at each successive 30 min, resulting in 48 measurements per day. Thermal gradients were achieved with a mean difference in temperature of 13.564 ºC between chambers 1 and 6 by simultaneously heating chamber 6 (mean temperature = 20.725 ± 0.712 ºC) and cooling chamber 1 (mean temperature = 7.161 ± 0.476 ºC). All temperatures were recorded each day at the same time of the day. The mean number of fish observed per day in each compartment + SD (n=30) was registered for each experimental group (see 2.2.2)

Additionally, in order to estimate the expression of TRPV and its relation with different temperatures, two group of fish (n = 12 for each group) were distributed in the first chamber of the thermal gradient tank, using a grid to block the centre hole in order to avoid connection between the chambers. Experimental groups for this assay consisted in an immersion challenge with IPNV ($^{+}$IPNV) group and a non-challenged control group. After 5 minutes of conditioning in the first chamber, 2 individuals were randomly sampled. The remaining individuals were allowed to move to the second chamber by removing the blocking grid and once the whole group was in the second chamber the hole was blocked again. Individuals were allowed to acclimatize for 5 minutes and another two fish were randomly sampled. The same procedure was followed for the next 4 thermal chambers. Sampled individuals were fixed and stored for further absolute qPCR analysis (see 2.7). Three independent experiments per group were carried out (Fig. 1).

2.4 ELISA measurement of plasma cytokine IL-1β, IL-6 and TNF-α and prostaglandin E_2

Blood plasma was obtained from individual salmon and stored at -80ºC until use. To determine the detection of IL-6, TNF-α and IL-1β in plasma samples of *Salmo salar*, indirect ELISA was performed according to Morales-Lange et al. (2015). Briefly, each plasma sample was diluted in carbonate buffer (60 mM NaHCO3, pH 9.6), planted (in duplicated for each marker) at 35 ng/µL (100 µL) in a Maxisorp plate (Nunc, Thermo Fisher Scientific, Waltham, United States) and incubated overnight at 4°C. After, each well was blocked with 1% Bovine Serum Albumin (BSA) for 2 h at 37 °C. Then, plates were incubated for 90 min at 37°C with the primary antibody

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anti-synthetic epitope (diluted en BSA) of TNF-α (diluted 1:500), IL-6 (diluted 1:500) and IL-1β (diluted 1:500). Later, the second antibody-HRP (Thermo Fisher Scientific, Waltham, United States) was incubated for 60 min at 37 °C in 1:7000 dilution. Finally, 100 µL per well of chromagen substrate 3,3',5,5'-tetramethylbenzidine (TMB) single solution (Invitrogen, California, United States) was added and incubated for 30 min at room temperature. Reaction was stopped with 50 µL of 1 N sulfuric acid and read at 450 nm on a VERSAmax microplate reader. Primary antibodies against cytokines were produced according Bethke et al. (2012). Synthetic epitope peptides were used for immunization in CF-1 mouse for IL-6 and TNF-α (Rojas et al., 2012) and New Zealand rabbit for IL-1β (Schmitt et al., 2015). For validation, antibody efficiency was determined by the calibration curve of the antibody against the synthetic peptide used for the immunization through indirect ELISA (Santana et al., 2013) and antibody specificity was verified by Western blot as described before in Schmitt et al. (2015). Measurement of plasma PGE_2 levels was carried out using a commercial monoclonal EIA according to the manufacturer's instructions (Cayman, Michigan, United States). This assay has a range from 8.6 - 2,000 pg/ml and a sensitivity (80% B/B_0) of approximately 30 pg/ml.

2.5. *In situ* hybridization

TNF-R1, EP3 receptor, TRPV1 and TRPV4 mRNAs were localized in the brain of ~1 year old salmon by *in situ* hybridization using digoxigenin-alkaline phosphatase (DIG-AP) with incorporated cRNA probes (Supplementary Table 1). Brains were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, dehydrated and embedded in paraffin (Sigma-Aldrich, Missouri, United States). Adjacent dorsal sections (7 µm) were washed twice with xylene, followed by decreasing concentrations of ethanol, and finally washed twice in Tris buffered saline containing 0.05% Tween-20 (TBST). Sections were prefixed with PFA for 20 min and then treated with proteinase K (0.5 µg/mL; Sigma-Aldrich, Missouri, United States) in TBS with $CaCl_2$ (2 mM) for 15 min at 37°C and fixed again for 5 min. Endogenous phosphatase activity was blocked by acetylation

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treatment (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0; Sigma-Aldrich, Missouri, United States) for 10 min with agitation. Samples were pre-hybridized in hybridization buffer (50% deionized formamide [Millipore, Massachusetts, United States], 2x SSC [Sigma-Aldrich, Missouri, United States], 1x Denhardt's solution [Sigma, Missouri, United States], 250 µg/mL yeast tRNA [Invitrogen, California, United States], 10% dextran sulphate [Millipore, Massachusetts, United States]) for 30 min-1 h in a humid chamber containing 2x SSC/50% formamide. Hybridization was carried out overnight at 60°C using 500-1000 ng/mL cRNA probes. To synthesize DIG-AP riboprobes, the PCR product was generated with specific primers for each mRNA containing the T3/T7 RNA polymerase promotor sequence (Supplementary Table 1). The sections were hybridized with antisense and sense (negative control) riboprobes. After hybridization, sections were washed in 2x SSC and incubated in 2x SSC/50% formamide for 30 min at 60°C, followed by washes in 2x SSC for 15 min at 60°C, twice in 0.1x SSC at 60°C, and finally in TBST. Pre-blocking was performed with 0.5% bovine serum albumin (BSA; Sigma-Aldrich, Missouri, United States) in TBST buffer for 1 h at room temperature and subsequently incubated with AP-conjugated anti-DIG antibody (Roche, Basel, Switzerland) 1:1000 diluted in TBST with 0.5% BSA overnight at 4°C. Color development was carried out by incubating the sections in Nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Roche, Basel, Switzerland) in the dark. After being air-dried, the slides were mounted using Faramount mounting medium (Dako). Pictures were taken with an Olympus AX70 microscope.

2.6. COX-2 immunolocalization by immunofluorescence microscopy

Brains were fixed in phosphate-buffered (PBS) 4% paraformaldehyde at 4°C overnight for COX-2 immunolocalization. After dehydration, samples were embedded in paraffin. Sections of 7 µm thickness were dewaxed with xylene and rehydrated through graded ethanol into water. Nonspecific antibody sites were blocked by pre-incubating slides with 5% bovine serum albumin (Sigma-Aldrich, Michigan, United States) in PBS at room temperature for 30 min. Then, sections

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were incubated with primary antibody goat anti-COX-2 (1:100; Cayman, Michigan, United States) (Feng et al., 2012) in the same blocking solution at 4°C overnight. After PBS washes, sections were incubated with Alexa Fluor 488 chicken anti-goat IgG secondary antibody (1:500; Life Technologies, California, United States) at room temperature for 90 min. Then, sections were washed in PBS and counterstained with propidium iodide (Sigma-Aldrich, Michigan, United States) and slides mounted with Vectashield anti-fading medium (VECTASHIELD®, Vector Laboratories, California, United States). Negative controls were obtained by pre-adsorption of the primary antibody with the blocking peptide (Cayman, Michigan, United States). Sections were examined and photographed with a Zeiss LSM 700 confocal microscope (with the same fluorescence intensity and exposure).

2.7 Absolute mRNA quantification by real-time polymerase chain reaction (qPCR)

2.7.1. RNA extraction and cDNA synthesis

All samples were snap-frozen in liquid nitrogen and conserved at -80 °C until further analysis. Total RNA was extracted from head kidney and spleen (100 mg) with the TRI Reagent® (0.5 mL; Sigma-Aldrich Missouri, United States) and was quantified by absorbance at 260 nm. Only samples with an A260/280 ratio between 1.8 and 2.1, and an A260/230 ratio above 1.8 were used for reverse transcription. Purified RNA integrity was confirmed by agarose denaturing gel electrophoresis. cDNA was synthesized from total RNA (200 ng/µL) using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) according to the manufacturer's indications.

2.7.2 Absolute mRNA abundance quantification

qPCR analysis was performed using the SsoAdvanced™ Universal SYBR® Green Supermix (2X) (Bio-Rad, California, United States). cDNA used in qPCR assays was first diluted with nuclease-free water (Qiagen, Hilden, Germany). Each qPCR mixture contained the

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SsoAdvanced™ Universal SYBR® Green Supermix , 2 µL cDNA, 500 nM each primer, and RNase-free water to a final volume of 10 µL. Amplification was performed in triplicate in 96-well plates with the following thermal cycling conditions: initial activation for 3 min at 98 °C, followed by 40 cycles of 15 s at 98 °C, 30 s at Tm °C, and 30 s at 72 °C. A dilution series made from known concentrations of plasmid containing the PCR inserts was used to calculate absolute copy numbers for each of the genes examined. Previously published primers were used (Supplementary Table 2).

2.7.3 Absolute quantification standards

An absolute quantification approach was used that involved calculating the number of gene copies in unknown "test" samples from comparison with a standard curve prepared using a dilution series of linearized plasmids with known concentrations (Pfaffl, 2004). The PCR product for each gene was extracted from agarose gel using the Nucleospin Gel and PCR Clean-Up Kit (MACHEREY-NAGEL, Dueren, Germany). The PCR amplicons were cloned the using pGEM-T Easy Vector and JM109 High-Efficiency Competent Cells (Promega, Madison, WI, USA). The Nucleospin Plasmid Quick Pure Kit (MACHEREY-NAGEL) was used to purify the plasmid DNA containing the PCR insert. Then, the plasmid was linearized using the HindIII restriction enzyme to prevent amplification efficiency problems that can arise from using supercoiled plasmids (Hou et al., 2010), and the amount of dsDNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, California, United States). The concentration of each plasmid was calculated by absorbance at 260 nm, and a five-fold dilution series produced for copy number calculations via qPCR and using Equation 1.

$$\boldsymbol{Number\ of\ copies} = \frac{\boldsymbol{amount} * \mathbf{6{,}022\times10^{23}}}{\boldsymbol{length}*\mathbf{1\times10^{9}}*\mathbf{650}} \text{ (Eq. 1)}$$

where the *amount* of DNA (ng) was derived from absorbance at 260 nm and *length* (base pairs) was determined by adding the PCR product length to the size of the plasmid.

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The use of these standard curves controlled for amplification efficiency differences between assays and permitted calculating the "absolute" number of mRNA transcripts, thereby facilitating gene comparisons.

2.8 Statistical Analyses

The behavioural fever analysis was carried out by pairwise comparisons between groups when the overall *p*-value was significant, using the Bonferroni method for adjusting the level of significance. Predictions of fish numbers from final models were calculated and plotted to evaluate interactions. All statistical analyses were performed with Stata version 14 (StataCorp LP). For gene expression after absolute mRNA quantification by real-time polymerase chain reaction (qPCR) and ELISA measurement of plasma cytokine IL-1β, IL-6 and TNF-α and prostaglandin E_2, all data was tested for normality and homogeneity of variances using the Shapiro-Wilk's and Levene's test respectively. When necessary, data were log10 transformed to achieve normality and variances homogenized. Data obtained was analysed using Statistica 6.0 software (Statsoft Inc., Tulsa, OK) and two-way ANOVA followed by Tukey HSD *post hoc* test for multiple comparisons. Results were considered significant when $p < 0.05$. Graphs were plotted with GraphPad PRISM v6.0 (GraphPad Software, Inc. California, USA).

3. Results

3.1. Establishment of behavioural fever

Two statistical analyses were carried out to understand behaviour fever in the four experimental groups. In the first analysis, the aim was to evaluate whether fish challenged with IPNV did prefer chambers with higher temperatures, while the second was designed to evaluate how fish were distributed across the 6 chambers, and to determine if challenge with IPNV affected this distribution (Fig. 1). In the first instance, we modelled the data using binomial generalized linear models, however, the dispersion statistics from these models suggested that variation in the

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data was smaller than the expected variability based on binomial distribution. Consequently, we moved to Generalized Poisson regression models (Hardin & Hilbe, 2012), which are recommended for under-dispersed data (Zuur et al., 2013). For the first analysis, we modelled the number of fish that were found in chambers 5 and 6 as a function of the two temperature groups (gradient and constant), the challenge groups (Control-Mock, RTR-IPNV, WTR-IPNV), and their interaction (4 groups in total). Likely temporal autocorrelation given by repeated measurements over time was controlled by including in the model the number of fish found in chambers 5 and 6 in the preceding observations. This analysis showed that under constant temperature conditions (Control-Mock and RTR-IPNV), the number of fish that preferred warm chambers (5 or 6) was the same for both treatments. In the thermal gradient groups (WTR), the number of fish found in chamber 5 or 6 was significantly higher for the IPNV challenged individuals in comparison to the control group ($p < 10^{-3}$) (Fig. 2A). The results suggest that the IPNV challenged group with access to a thermal gradient tank preferably chose the warm chambers (nº 5 and 6) and the unchallenged fish prefer chamber 3 (thermo-preferendum, Wald test $p < 10^{-3}$). Predictions of fish numbers from final models were calculated and plotted to evaluate interactions (Fig. 2B).

3.2. Expression of behavioural fever is damaging for IPNV transcription

The influence of behavioural fever on viral transcription was examined by qPCR with primers (WB117) and Universal ProbeLibrary probes (UPL) specific for the VP2 segment of the IPN virus. Total RNA was isolated from head kidney and spleen at 24 h after immersion challenge with IPNV or virus free cell culture supernatant (Fig. 3). The absolute expression of VP2 transcripts was higher in *S. salar* maintained at constant temperature (RTR) in comparison to the individuals in the thermal gradient (WTR). The results are representative of three independent experiments (n=10 by experiment x three replicate, N=90).

3.3. Molecular mechanisms of behavioural fever induction

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3.3.1 Exogenous and endogenous pyrogens

In endothermic vertebrates fever is driven by peripheral pro-inflammatory cytokines produced in response to pathogens and by subsequent prostaglandin (PGE_2) synthesis. To test if these molecules also might act as endogenous pyrogens for behavioural fever in fish absolute mRNA abundance of cytokines (IL-1β, IL-6, TNF-α and IFN-γ), cyclooxygenase 2 (COX-2) and microsomal PGE synthase 1 (PGD_2) was measured in head kidney and spleen in all experimental groups (control, WTR and RTR). As shown in Fig. 4, an increase in mRNA abundance of IL1-β was registered in individuals challenged with the virus and with access to a thermal gradient (WTR-IPNV, Fig. 3, $p < 10^{-3}$) in contrast to that observed in individuals with no access to a thermal gradient (RTR-IPNV). TNF-α, IL-6, COX-2 and mPGES-1 mRNAs did not show statistical differences between challenged groups (RTR-IPNV – WTR-IPNV, $p > 10^{-3}$). In head kidney, only IFN-γ did not show any statistical differences when compared with the other experimental groups ($p < 10^{-3}$). However, IFN-γ showed a significant increase ($p < 10^{-3}$) in the spleen of the WTR-IPNV experimental group. Even although ELISA assays show that plasmatic levels of these proteins were higher in the challenged groups (WTR-IPNV and RTR-IPNV), significant differences were only observed for IL-1β and IL-6 ($p < 10^{-3}$) in comparison with the non-challenged control group (Fig. 5). Our results evidence that the viral stimuli trigger endogenous pyrogen production including the pro-inflammatory cytokines IL-1β, IL-6 and prostaglandin mPGES-1 and increased temperature (WTR vs RTR) promotes changes in the rate/synthesis/release of cytokines and/or mRNA abundance, suggesting a synergistic action between in temperature and immune system activation.

3.3.2 Brain cytokine receptors and TRPV channels during behavioural fever.

To explore the homology between the behavioural fever pathway and previously reported pathways in endotherms immunofluorescence microscopy, in situ hybridization and absolute mRNA quantification of the receptors, IL-1r, IL-6r, TNF-R1, EP3 receptor and TRPV channels

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TRPV1 and TRPV4 in the brain of IPNV-challenged fish, with or without access to the thermal gradient was carried out. No significant differences were observed for the TNF receptor (TNF-R1) when comparing absolute mRNA abundance between the experimental groups ($p > 10^{-3}$). IL-1 receptor (IL-1r) mRNA abundance was statistically higher ($p < 10^{-3}$) in the challenged group with no access to a thermal gradient (RTR-IPNV) whereas a significant increase in mRNA abundance was recorded for the IL-6 receptor (IL-6r) in both challenged groups with and without access to the thermal gradient when compared to control (Fig. 6). Quantification of COX-2 mRNA abundance showed a significant increase in the challenged group with access to the thermal gradient (WTR-IPNV, $p < 10^{-3}$) when compared to other groups (Fig. 6). For PGE_2 receptor (EP3 receptor), a significant increase in mRNA copy number was also observed in the challenged group with access to a thermal gradient (WTR-IPNV, Fig. 7). TRPV1 mRNA abundance was significantly higher in WTR individuals challenged with IPN virus ($p < 10^{-3}$) mainly at extreme cold and warm temperatures (chambers 1-2 and 5-6). The TRPV1 mRNA abundance decreased from chambers 3 to 4 when individuals were closer to their thermal preferendum (15°C). These results might suggest that TRPV1 expression could be activated by extreme temperatures, rather than the virus infection itself. Additional experiments are required to robustly test this hypothesis.

A different pattern was registered for TRPV4, where significant differences were registered within each chamber when comparing challenged and non-challenged groups. IPNV challenged groups showed lower mRNA abundance of TRPV4 compared to the non-challenged groups, specifically in the final chambers (18 and 20 °C). This suggests that TRPV4 expression may be inhibited in the presence of a virus, allowing individuals to move to warmer regions (Fig. 8). In addition, our results show for first time localization of TRPVs, EP3 receptor and COX-2 induced after viral infection in fish the brain. *In situ* hybridization analysis shows that TRPV1 and TRPV4 and EP3 receptor transcripts were only detected in the telencephalon and optic lobe (Fig. 9 and 10). Furthermore we carried out immunolocalization of the COX-2 protein and confocal

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microscopy analysis revealed the localization of COX-2 mainly to specific areas of the telencephalon, optic lobe and cerebellum (Fig. 11).

4. Discussion

The present findings demonstrate that the prostaglandin brain receptor, EP3 receptor, and TRP channels might be involved in the behavioural fever response induced by the pro-inflammatory cytokines and prostaglandin PGE_2 activated by the response to the virus. Our findings show that TNF-α, IL-1β, IL-6 release and COX-2 activation in the brain could be involved in behavioural fever in *Salmo salar*. In addition, our findings also show that the febrile response triggered by infectious pancreatic necrosis virus (IPNV) is linked to the regulation of TRP channels. To our knowledge these novel results identify a tentative linkage between the regulation of TRP thermal receptors, directly activated by hot or cold temperature range, via EP3 receptor activation and PGE_2 during behavioural fever.

We recently demonstrated several underlying mechanisms that contribute to the development of behavioural fever in zebrafish challenged with the PAMP, dsRNA and the spring viraemia of carp virus (Boltaña et al., 2013). Here we extend this finding by showing the mechanistic basis of induced behavioural fever in response to infectious pancreatic necrosis virus (IPNV) in salmon. The virus, like other pathogens are not directly responsible for the development of fever that itself is generated as a consequence of PAMP-PRR interactions in the immune system leading to the synthesis of endogenous pyrogens, such as cytokines (Roth & Blatteis, 2014; Roth & de Souza, 2001). The involvement of cytokines in fever has been widely demonstrated in mammals but not in ectotherms (Janský et al., 1995; Kluger, 1991; LeMay et al., 1990; Roth et al., 1993). Here, we first show that the febrile response to IPNV challenge is triggered by several blood-borne cytokines, IL-1β, IL-6 and TNF-α. As in mammals (Evans et al., 2015), IL-6 in ectotherms appears to have a highly similar role. It has been reported that of all cytokines measurable in blood plasma IL-6 has a high correlation with temperature changes caused by fever,

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and its expression is, in part, under the control of other pyrogens or cytokines such as IL1-β and TNF-α (LeMay et al., 1990; Roth et al., 1993; van Damme et al., 1986).

In addition to the above mentioned cytokines that are not themselves the final mediators of fever as their pyrogenic activity is dependent upon the synthesis of other mediators such as the prostaglandins (Nadjar et al., 2005; Pecchi et al., 2009; Rakus, Ronsmans, & Vanderplasschen, 2017; Rummel et al., 2006; Rummel et al., 2005). Therefore we investigated the involvement of the EP3 receptor in the fever response induced by PGE_2 and COX-2 as central mediators promoting febrile responses (Banks, 2005; Evans et al., 2015). A rise in COX-2 mRNA abundance in the brain of IPNV challenged animals with access to behavioural fever was observed that was in stark contrast to those without access to a thermal gradient (RTR). On the other hand, the COX-2 mRNA increase might be induced by IL-6, IL-1β, or TNF-α, clearly showing that prostaglandin synthesis also is involved in behavioural fever in ectotherms. IL-1β and IL-6 receptors (IL-1r and IL-6r) which mediate the synthesis of COX-2, have been identified in the preoptic nucleus region of the hypothalamus (Cao et al., 1996; Konsman et al., 2004). Our results in individuals displaying behavioural fever in the brain demonstrate that IL1r mRNA transcript abundance in the POA also increases in response to the virus however co-localisation studies are required to identify the cell type involved.

Cooperation between the IL-6 receptor (IL-6r) and EP3 receptors is critical for the progress of PGE_2 induced fever (Coleman et al., 1994; Lazarus et al., 2007; Ushikubi et al., 1995). Previous studies have demonstrated that IL-6r expression is induced under immune challenge in several brain regions (Gadient & Otten, 1996; Vallières & Rivest, 1997). Eskilsson et al (2014) demonstrated that when IL-6 is endogenously released during a systemic inflammation, it acts as a pyrogen when linked to IL-6r in endothelial cells in the brain inducing PGE_2 synthesis in a process that probably involves the action of other peripheral cytokines. Our results show increased IL-6 and PGE_2 levels and also a concurrent increase in IL-6r receptor mRNA abundance in the brain

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thus supporting previous observations (Eskilsson et al., 2014) and suggesting that IL-6/IL-6r may have a key functional role in the development of behavioural fever in ectotherms.

Although it is not clear how prostaglandin is responsible for the induction of behavioural fever in ectotherms, our results, together with the findings from Dhaka et al. (2006), suggest that changes in thermoregulatory behaviour could be mediated by the activation of transient receptor potential channels (TRP) (Gavva et al., 2007; Hori, 1984; Jancsó-Gábor et al., 1970; Swanson et al., 2005). Several studies have suggested a key role of TRP channels in temperature sensing and thermoregulation in endotherms (Gavva et al., 2007; Hori, 1984; Jancsó-Gábor et al., 1970; Swanson et al., 2005). In mammals for example, TRPM2 (transient receptor potential channel M2) ion channels function as ionotropic warm receptors in hypothalamic neurons to limit the fever response (Song et al., 2016). Other thermally sensitive TRP ion channels that also participate in the regulation of fever are the TRPV1 splice variants (Zaelzer et al., 2015). In the present study, we hypothesized that TRP channels may be orchestrating the regulatory mechanisms by which mobile ectotherms can modify their temperature requirements and in consequence their thermoregulatory behaviour during an infection (Garrity et al., 2010; Morrison & Nakamura, 2011).

Considering our previous findings that PGE_2 is involved in behavioural fever (Boltaña et al., 2013) and the present data showing the involvement of endogenous pyrogens such as TNF-α, IL-1β, IL-6, and their ability to induce COX-2, PGE3 receptor and prostaglandin synthesis, a new central thermal-sensing mechanism for fever development could be hypothesized where TRP channels (TRPV1 and TRV4) and PGE_2 collaborate to regulate the development of behavioural fever in response to an exogenous stimulus. Our results suggest that mRNA encoded by TRPV1-TRPV4 are tightly linked to thermal sensing during behavioural fever. In mammals it has been reported that the TRPV4 ion channel found in the POA it is key element of thermoregulatory mechanism, where it activation decreased body temperature whereas and the blockade drives hyperthermia (Güler et al., 2002; Watanabe et al., 2002; Yadav et al., 2017). Our results support

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these observations and show that regardless of the temperature, after viral challenge, there is a significant decrease in TRPV4 mRNA abundance. This may contribute to an altered thermoregulatory behaviour by promoting movement to warmer temperatures. We suggest that once immune system recognition systems are activated by the virus the synthesis of endogenous pyrogens including PGE_2, IL-6 and IL-1β is activated driving a decrease in TRPV4 mRNA abundance. This mechanism is linked to the thermo-sensing behavioural mechanism which re-adjusts and promotes increased preference towards significantly warm temperatures therefore promoting the development of behavioural fever. Once behavioural fever has been triggered and PGE_2 abundance has increased, the activation of TRPV1 might act as a "warning" sign to maintaining thermal homeostasis and thus preventing physiological harm associated with the prolonged exposure to hot temperatures. In fish, TRPV1 activation also regulates heat-evoked locomotion triggering the avoidance of extreme temperatures allowing the discrimination between noxious and inoffensive temperatures (Gau et al., 2013). Similar studies also suggest a rapid activation of TRPV1 channels by noxious heat (> 43°C) (Caterina et al., 1997; Tominaga et al., 1998). Studies in mammalian models support our results and suggest that thermoregulation during febrile processes is regulated by a dynamic and permissive modulation between TRPV1 and TRPV4 ion channels in the magnocellular neurosecretory neurons (Sudbury & Bourque, 2013). In mobile ectotherms thermoregulation during behavioural fever may be mediated by TRP channels, however further experimentation addressing function are required to robustly test this hypothesis

5. Conclusion

This novel study casts light upon the molecular mechanisms underpinning behavioral fever in ectotherms and particularly in fish. We have shown that a virus may act as an inducer of pyrogenesis via behavioural fever by promoting the synthesis of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. In turn these proteins, particularly IL-6, interact with specific brain receptors (IL6r) enhancing local synthesis of COX-2, mPGES-1and PGE_2 release. This mechanism acts in

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synergy with central nervous system mechanisms in response to infection and temperature sensing. The thermal channels TRPV1 and TRPV4 may act to coordinate acute changes in temperature preference promoting displacement through a thermal gradient allowing the development of behavioural fever and a subsequent return to thermal homeostasis. In the current study evidence is proposed for a role of TRPV channels and COX-2 - PGE_2 in the development of behavioural fever in mobile ectotherms however further functional assays are required. This may include for example pharmacological inhibition of COX-2, quantification of TRPV channel responses using iRNA-based and conventional pharmacological inhibitors, or gene-editing mechanisms applying CRISPR/CAS9 methodology in future studies. The fact that the "*fever*" response to infection and injury has been maintained throughout at least 600 million years of evolution strongly suggests that the mechanistic bases of behavioural fever in ectotherms and fever in endotherms are strongly conserved. Although our results suggest evidence of a conserved pathway of behavioral fever in response to virus, there still remains a lack of in-depth knowledge regarding the underlying functional mechanisms involved in fever.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Figure captions

Fig. 1 Experimental design and thermoregulatory limits. Experimental design diagram showing both thermoregulatory treatments (i.e. restricted thermoregulatory range; Δ_T 1.4 °C and wide

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thermoregulatory range; Δ_T 6.4 °C) for *S. salar* rearing. Temperature treatments are color-coded, and the experimental duration for each developmental period is indicated in horizontal grey boxes.

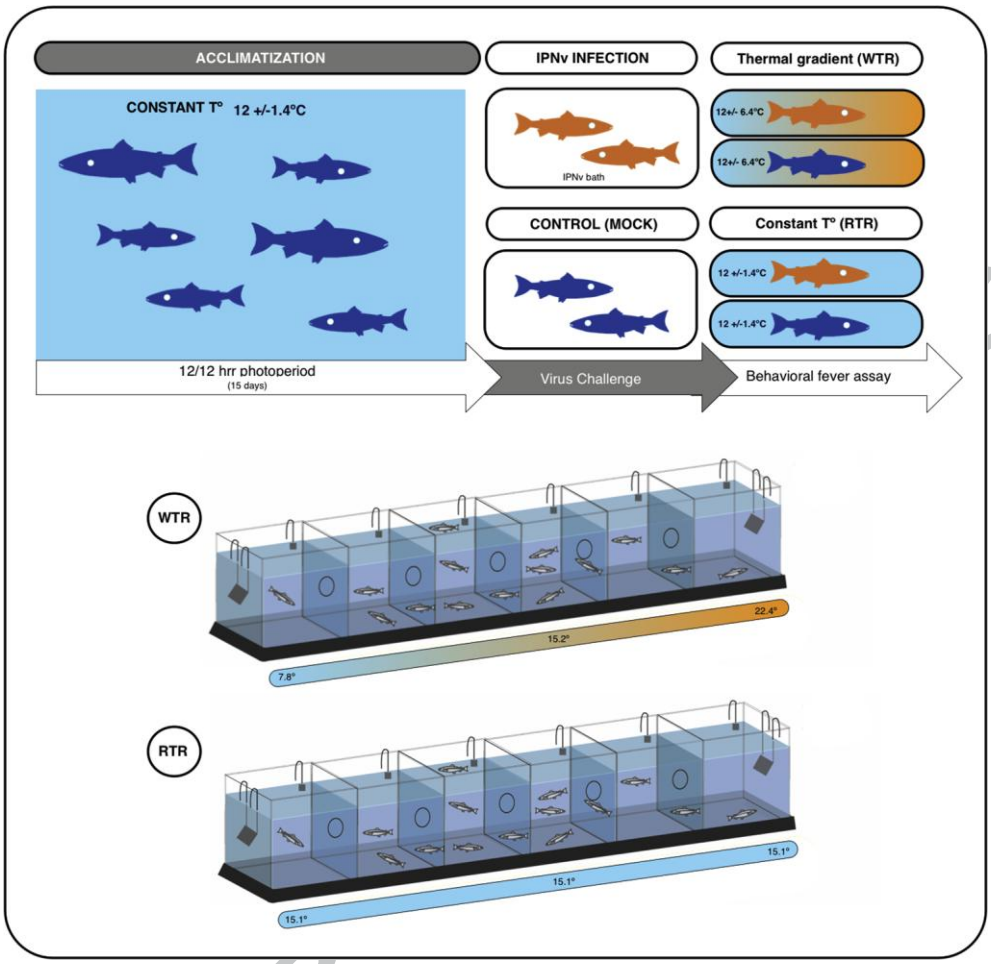

Fig. 2 Behavioural fever registered in IPNV-treated and non-challenged (Mock) *Salmo salar* under two thermal conditions (thermal gradient and constant temperature). (A) Frequency of individual occupation in hot chambers (5 or 6). (B) Fish distribution among the 6 chambers (n = 10 individuals. *** indicates significant differences, $p < 0.05$).

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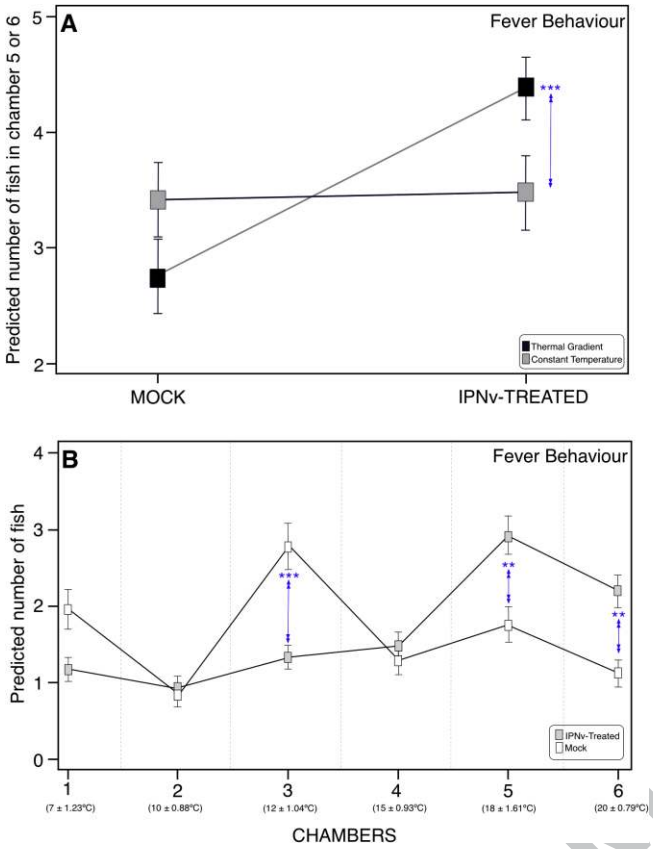

Fig. 3 Viral load expressed as the abundance of VP2 segment of the IPNV virus by qPCR for head kidney and spleen. The experimental groups are: virus free (Control), IPNV challenged at constant temperature (RTR^+, grey color bar) and IPNV challenged in thermal gradient tank "fever group" (WTR^+, white color bar). Different letters denote significant differences between groups. Values are represented as the mean VP2 copy number ± SD. Different letters denote significant differences between experimental groups (2way ANOVA; $P < 0.05$; $p < 0.0001$).

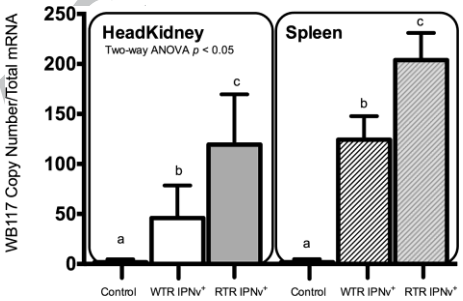

Fig. 4 Expression profile of pro-inflammatory cytokines IL1-β, IL-6, TNF-α, IFN-γ, COX-2 and mPGES-1in head kidney and spleen of *Salmo salar*. Values are represented as the mean mRNA abundance ± SD. The experimental groups are: virus free (Control), IPNV challenged at constant temperature (RTR^+, grey color bar) and IPNV challenged in thermal gradient tank "fever group"

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(WTR$^+$, white color bar). Different letters denote significant differences between groups (2way ANOVA; P < 0.05; p < 0.0001).

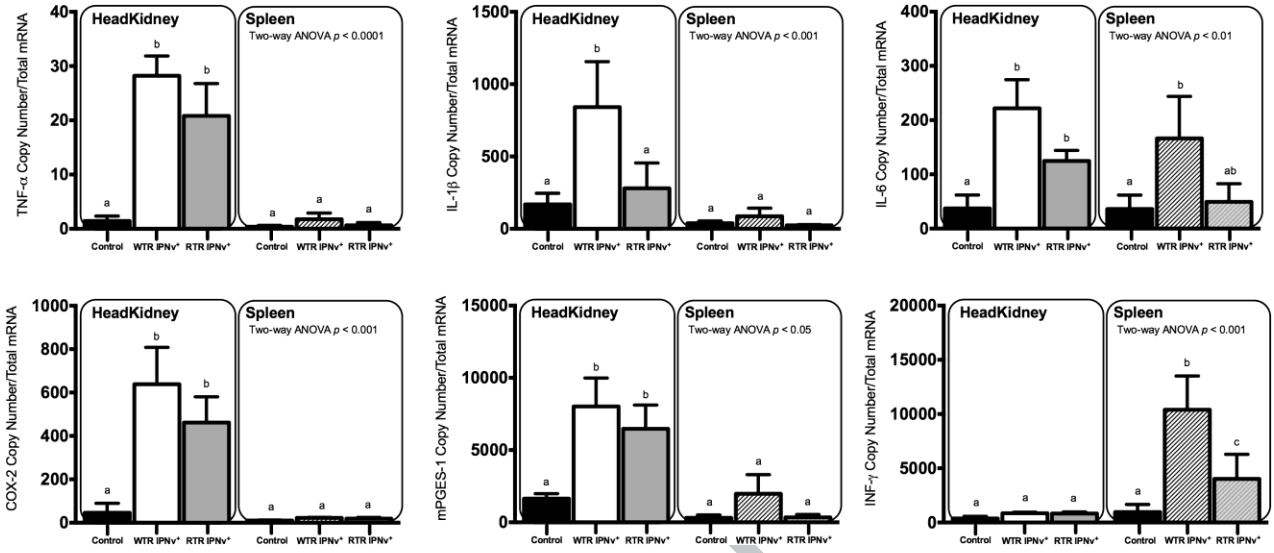

Fig. 5 *Salmo salar* plasma concentration (pg*mL^{-1}) of IL1-β, IL-6, TNF-α and PGE_2. Values are represented as the mean protein concentration ± SD. Values are represented as the mean mRNA abundance ± SD. The experimental groups are: virus free (Control), IPNV challenged at constant temperature (RTR$^+$, grey color bar) and IPNV challenged in thermal gradient tank "fever group" (WTR$^+$, white color bar). Different letters denote significant differences between groups (2way ANOVA; P < 0.05; p < 0.0001).

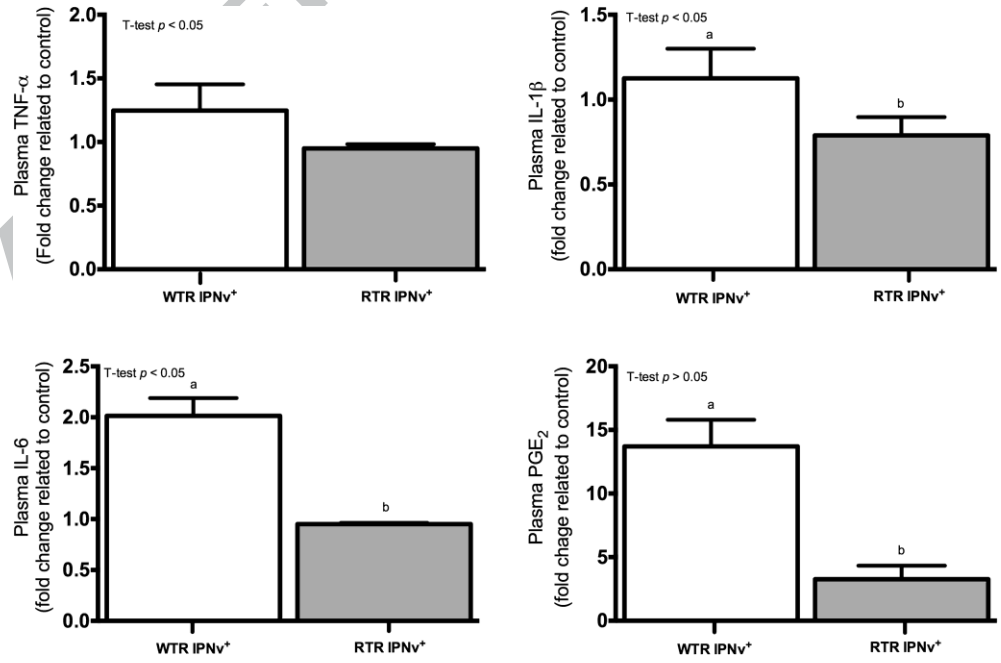

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Fig. 6 *Salmo salar* brain expression profile of cytokine and prostaglandin receptors in the brain (IL-1r, IL-6r, TNFr and EP3 Receptor). Values are represented as the mean mRNA abundance ± SD. The experimental groups are: virus free (Control), IPNV challenged at constant temperature (RTR^+, grey color bar) and IPNV challenged in thermal gradient tank "fever group" (WTR^+, white color bar). Different letters denote significantly different mRNA levels between groups (2way ANOVA; $P < 0.05$; $p < 0.0001$).

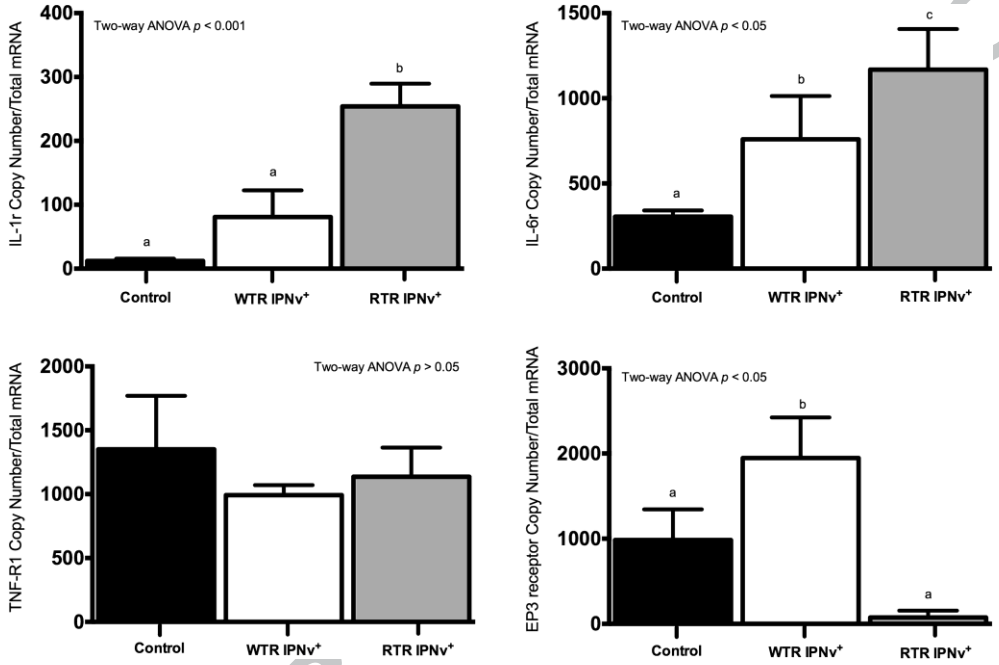

Fig. 7 *Salmo salar* brain expression profiles of COX-2 and mPGES-1. Values are represented as mean mRNA abundance ± SD. The experimental groups are: virus free (Control), IPNV challenged at constant temperature (RTR^+, grey color bar) and IPNV challenged in thermal gradient tank "fever group" (WTR^+, white color bar). Different letters denote significantly different mRNA levels between groups (2way ANOVA; $P < 0.05$; $p < 0.0001$).

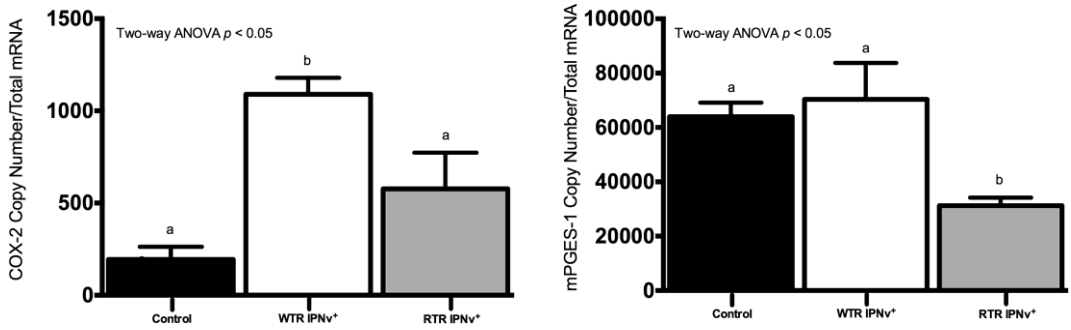

Fig. 8 *Salmo salar* brain expression profiles for thermal receptor A) TRPV1 and B) TRPV4.

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Values are represented as the mean mRNA abundance ± SD. Values are represented as the mean mRNA abundance ± SD. The experimental groups are: virus free (Control, white color bar), and IPNV challenged in thermal gradient tank "fever group" (WTR^{+}, grey color bar). Different letters denote significantly different mRNA levels between groups (2way ANOVA; $P < 0.05$; $p < 0.0001$).

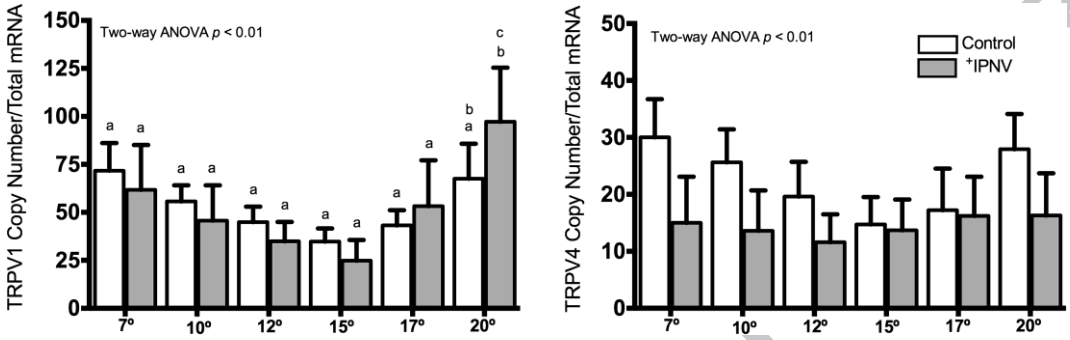

Fig. 9 *In situ* hybridization of TRPV1 and TRPV4 transcripts in the *Salmo salar* brain. A) Schematic diagram showing the different brain regions: (C) Cerebellum, (OT) Optic lobe, (T) Telencephalon and (OB) olfactory bulb. B-E) Negative controls (sense riboprobes) for both TRPV1 and TRPV4 transcripts were performed showing no specific staining. F-Q) mRNA expression localization of TRPV1 and TRPV4 transcripts in different brain regions. Scale bar represents 25 µm.

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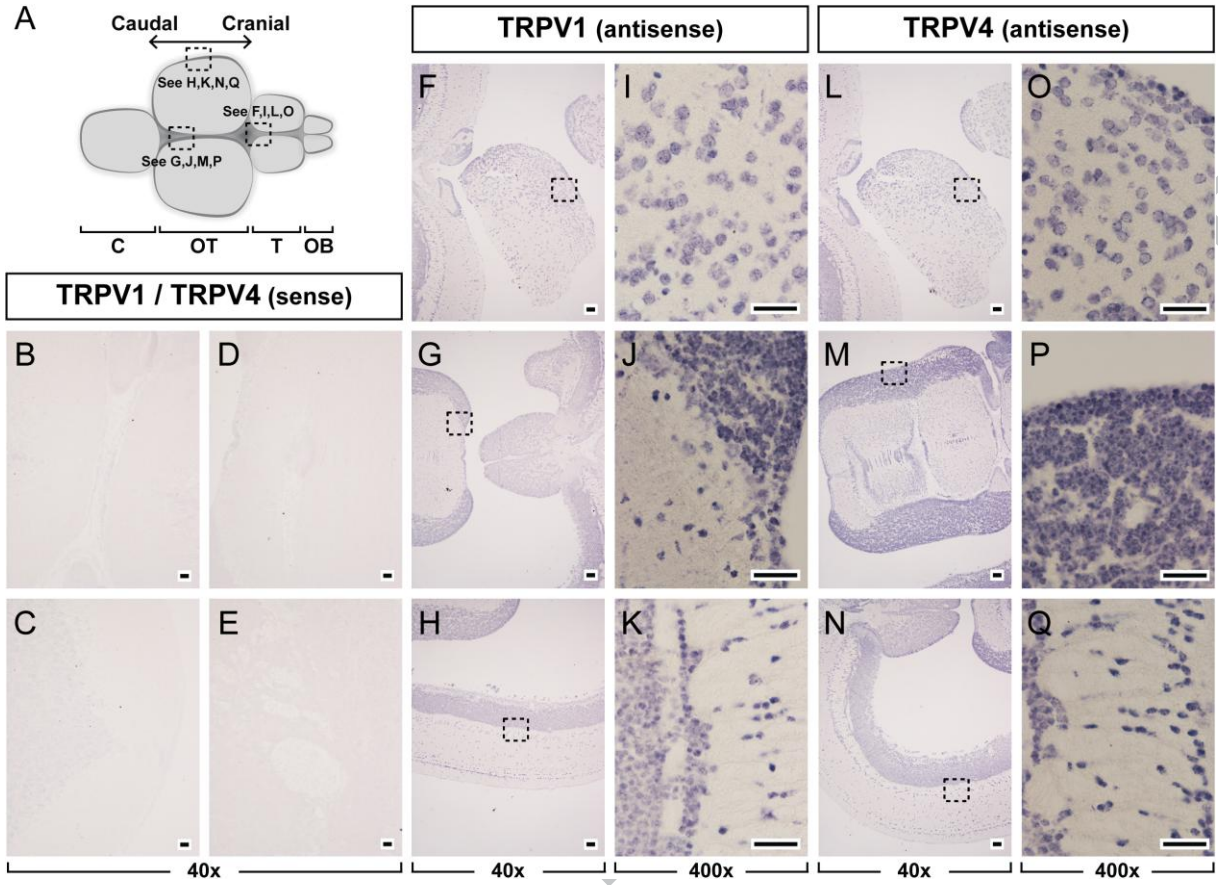

Fig. 10 *In situ* hybridization of EP3 receptor transcripts in the *Salmo salar* brain. Left panel shows the different brain regions: (C) Cerebellum, (OT) Optic lobe, (T) Telencephalon and (OB) olfactory bulb. A-J) mRNA expression localization of EP3 receptor transcripts in different brain regions. K-L) Negative controls (sense riboprobes) were performed showing no specific staining. Scale bar represents 25 µm.

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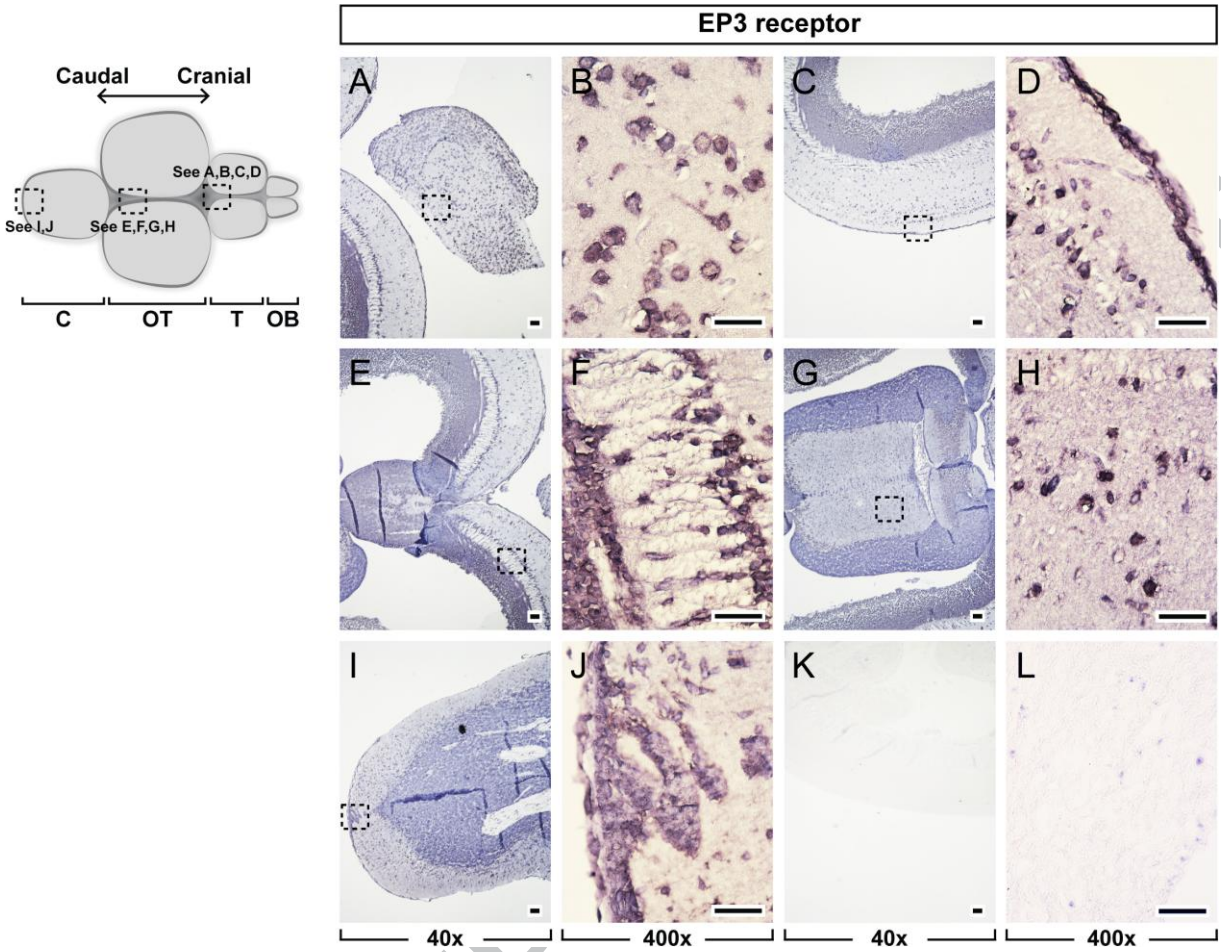

Fig. 11 Immunolocalization of COX-2 protein in the *Salmo salar* brain by fluorescence microscopy. Left panel shows the different brain regions: (C) Cerebellum, (OT) Optic lobe, (T) Telencephalon and (OB) olfactory bulb. A-C) Negative controls (pre-adsorbed antibody with the blocking peptide) were performed showing no specific staining. Red staining represents DNA (propidium iodide counterstain) and green staining represents COX-2 protein. Scale bar represents 25 µm.

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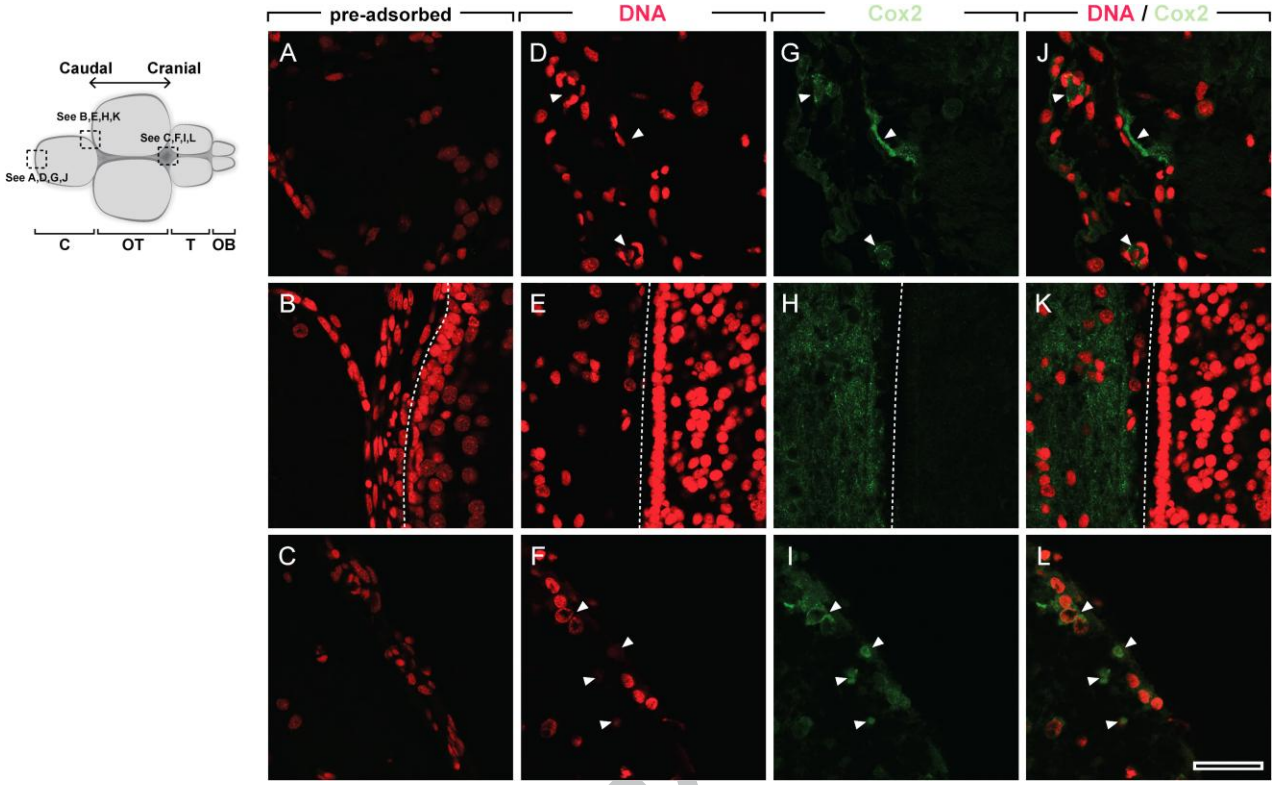

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Highlights

- Association between fever biomarkers and brain measures was examined.
- High IL-6 mRNA abundance and high IL-6 plasmatic concentration was associated with induction of COX-2 and EP3 receptor in brain.
- TRPV1 and TRPV4 have an extensive expression in the brain, especially during behavioural fever.

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