General and Comparative Endocrinology 275 (2019) 65–72

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Fish pituitary show an active immune response after *in vitro* stimulation with *Vibrio* bacterin

Xiaohong Liu[a,b], Ali R. Khansari[b], Lluis Tort[b,*]

[a] Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), Key Laboratory of Aquatic Science of Chongqing, School of Life Sciences, Southwest University, Chongqing 400715, China

[b] Department of Cell Biology, Physiology and Immunology, Universitat Autonoma de Barcelona, 08193 Barcelona, Spain

ARTICLE INFO

Keywords:
Pituitary
Vibrio bacterin
Immune response
Endocrine response
In vitro response
Trout

ABSTRACT

The pituitary is a central organ of the neuro-endocrine system in fish that plays critical roles in various physiological processes, including stress response and behavior. Although it is known that pituitary hormones can have a direct or indirect influence stimulating or suppressing the immune responses, whether there is a local immune response in the pituitary or what is the effect of the immune stimulus on the pituitary function in fish is unknown. With the aim to understand the interaction between the immune responses and the endocrine axes at the pituitary level, particularly the Hypothalamus-Pituitary-Interrenal (HPI) axis, pituitaries of rainbow trout (*Oncorhynchus mykiss*) were cultured *in vitro*, incubated with bacterin, or bacterin plus CRH, cortisol, human recombinant IL1β, or spleen medium for 3 h, and then genes involved in pro-inflammation (*il1β*, *il8*, *tnfα1*, *ifnγ*), anti-inflammation (*tgfβ1b*, *il10*), immune modulation (*mhcIIa*, *c3*, *mif*) and stress response (*crhbp*, *pomca*, *pomcb*, *gr1*) were tested. Data showed that, incubation with bacterin alone and bacterin plus recombinant IL1β or CRH, as well as medium from bacterin treated spleen caused significant up-regulation of pro-inflammatory genes *il1β* and *il8*, while down-regulated the anti-inflammatory gene *tgfβ1b*. Besides, recombinant IL1β plus bacterin or alone caused raise of *mhcIIa* and *tnfa*, respectively. On the contrary, just a slight or even no alteration was recorded in the expression of stress response genes including *crhbp*, *pomca*, *pomcb* and *gr1* in the *in vitro* cultured trout pituitary following this stimulation. These results suggest a local immune gene equipment in the pituitary of fish, and the potential for fish pituitary to develop both innate and adaptive immune responses, whereas that immune stimulation was not able to evoke a significant endocrine stress response *in vitro*.

1. Introduction

The pituitary, a main organ for hormone production is a central element of the endocrine system in vertebrates. Besides its function in the endocrine system, the pituitary gland has long been supposed to be involved in immunomodulation. On one hand the investigation of the endocrine regulation on the immune system is abundant, and it is widely accepted that various hormones such as growth hormone, prolactin, somatolactin (characterized in fish only), or follicle stimulating hormone play a role in the proliferation of immune cells (Blalock and Weigent, 1994), and the pituitary derived hormones are involved in axes such as HPA with adrenal glands, HPG with gonads, or HPT with thyroid hormones, thus participating in immune plasticity as well (Hodkinson et al., 2009; Bellavance and Rivest, 2014). On the other hand, a local immune response has been demonstrated to be present in mammalian pituitary function, but there is a lack of reports in comparative physiology. Thus, some cytokines such as IL1β, TNFα, IL6 and their receptors, as well as complement C3α, C5α and their receptors (Arzt, 2001; Utsuyama and Hirokawa, 2002; Francis et al., 2003, 2008) have been detected in the pituitary of rats or mice, and transcripts of IL6 family cytokines (IL6, LIF, IL11, CNTF) and their corresponding receptors are expressed in both normal and adenoma subtypes of human pituitaries (Hanisch et al., 2000). Moreover, expressions of cytokine receptors including IL6R, TNFαR, IFNγR and IL2R are reported to be induced in mice pituitary after LPS stimulation (Utsuyama and Hirokawa, 2002). Therefore, the immuno-modulatory activities of pituitary gland would act not just on the control of immune cells outside the pituitary, but also on the pituitary itself. All above-mentioned data suggest an active local immune system within the pituitary of mammals and that the pituitary is able to develop immune responses in mammals. Further investigation in mammals has shown that folliculo-stellate cells, the non-hormone pituitary producing cells,

* Corresponding author.
E-mail address: lluis.tort@uab.es (L. Tort).

https://doi.org/10.1016/j.ygcen.2019.02.011
Received 29 October 2018; Received in revised form 25 January 2019; Accepted 8 February 2019
Available online 12 February 2019

which are characterized by *S100b* expression, contain a population of cells that express dendritic cell markers such as *CD11b* and *MHCII* in rat pituitary, and these stellate cells have been recognized as the main source of inner pituitary cytokines (Glennon et al., 2015). Due to the expression of cytokines receptors in both endocrine and non-endocrine cells of pituitary, the endogenous cytokines have been proposed to modulate both directly and indirectly ACTH, cortisol and their related immune effects (Silverman et al., 2005). Besides, cytokines, particularly the IL1 produced outside the pituitary, can affect the HPA axis either directly at the different levels of this axis, or indirectly via promoting the production of prostaglandin E2 that mediates fever activation in the hypothalamus, thus stimulating the paraventricular nucleus PVN-CRF cells to activate the HPA axis (Dunn, 2007).

Among teleosts, a huge fish category, despite some basic similarities, there are many specific or particular characteristics regarding both structure and physiology. Although the investigation on the local immune response of the pituitary in fish is scarce, some available data showed that IL1RI (interleukin 1 receptor 1) can be detected in the pituitary of common carp (*Cyprinus carpio*), and IL1β can target melanotrope cells to induce the release of α-MSH (alpha-melanocyte stimulating hormone) and acetylated β-endorphin (Metz et al., 2006). What's more, a stellate cell network with expression of follistatin was reported in the tilapia pituitary (Golan et al., 2016), which suggested an active immune system located in the pituitary of fish, similarly to mammals. However, the non-expression of S100b in tilapia stellate cells (Golan et al., 2016) implied a difference between pituitaries of mammals and fish. In order to confirm the hypothesis that fish pituitary is able to induce an immune transcriptomic response after immune stimulation and therefore that there is an active local immune response in the fish pituitary, we investigated such interaction under *in vitro* conditions. Thus, pituitaries of rainbow trout (*Oncorhynchus mykiss*) were dissected and incubated with various stimulators for 3 h, and then the transcription of pro- and anti-inflammatory cytokines, immune regulators, and genes involved in the stress HPI axis were tested, thus trying to assess the roles of pituitary in the interactions between immune and endocrine systems in fish.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) (body weight of 71.95 ± 15.27 g) were obtained from a local fish farm (TroutFactory, Peramola, Spain). Fish were transferred to the Universitat Autonoma de Barcelona (UAB) fish facility (AQUAB). Fish were kept at 17 °C into a water circulating system, under the 12:12 h light: dark photoperiod. Fish were fed once per day at 10:00 am with a commercial diet. All fish were acclimatized for at least 2 weeks before the experiment, and during this period, no clinical signals of disease, malformation or injuries were observed. All experimental procedures were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international European Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2. Pharmacological agents

Cortisol (Hydrocortisone; Sigma cat #H2882-1G), Corticotropin Releasing Factor (CRH) human, rat > 95% (HPLC) (Sigma-Aldrich, cat #C3042) and Human recombinant Interleukin 1β (IL1β, expressed in *E. coli*, Sigma-Aldrich, cat# I9401) were dissolved according to manufacturer's instructions. The concentration of cortisol (100 ng/mL), CRH (100 nM), IL1β (50 ng/mL) used in the present study was chosen based on the previous studies (Hong et al., 2001; Katoh et al., 2004).

2.3. Vibrio anguillarum bacterin

ICTHIOVAC R VR (HIPRA, Spain) was the source for the inactivated *Vibrio anguillarum*. The composition consists of inactivated *V. anguillarum* bacterin, serotypes O1, O2a (the most pathogenic) and O2b with relative percent survival (RPS) 60%. 1 mL of inactivated *V. anguillarum* bacterin was isolated by a first centrifugation using 10,000 g for 10 min at 4 °C, washing twice using Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA), and then the pellet was resuspended by 1 mL of DMEM to get the stock solution. Different dilutions (1:20, 1:80, 1:400, 1:1500) after 1 h and 3 h incubation were then tested in order to determine the bacterin working solution. The 1:50 dilution with 3 h incubation was chosen based on the preliminary cytokine expression results.

2.4. Pituitary preparation and treatments

After lethal anesthesia using MS222 (Sigma-Aldrich, USA), the blood of each rainbow trout was extracted with a syringe from the caudal vein, and the pituitary of each fish was immediately dissected and separately placed in the 24-well plate, which was filled with 1 mL pre-cold DMEM (serum-free) in each well. In total, 60 pituitaries were collected, and they were randomly divided into 2 groups, one (non-bacterin group) treated without bacterin, and the other one (bacterin group) treated with 1:50 diluted bacterin. Each group was subdivided into 5 subgroups, named control (CTRL, pituitaries were treated with and without 1:50 diluted bacterin in no-bacterin and bacterin groups, respectively), IL1β (IL1, pituitaries were treated by an additional 50 ng/mL human recombinant IL1β in both groups), supernatant (SpM, pituitaries treated with medium from the *in vitro* cultured control and bacterin treated trout spleen in no-bacterin and bacterin groups, respectively, and no more bacterin was added in bacterin group), CRH (pituitaries treated with an additional 100 nM CRH in both two groups), Cortisol (CO, pituitaries treated with an addition of 100 ng/mL cortisol in both groups). There were 6 pituitaries per subgroup. After pituitary collection, plates were incubated for 1 h under the conditions of 17 °C, 5% CO_2 and 95% humidity. Then, the pharmacological agents and/or the bacterin were added into each subgroup, respectively, and incubated for another 3 h.

The medium from the *in vitro* cultured spleen used for bacterin-treated and control subgroups was prepared beforehand as follows: after the anesthesia overdose, the spleens of trout were excised and cut into small pieces; after washing by DMEM twice, these tissues were then cultured in the DMEM (serum-free). For the medium used in supernatant subgroups of bacterin and no-bacterin groups, the spleen tissue pieces were cultured by DMEM supplied with and without 1:50 diluted bacterin, respectively. After 3 h incubation under 17oC, 5% CO2 and 95% humidity, the medium was separately collected, centrifuged at 10,000 rpm for 10 min to remove the tissues and bacterin cells, and then the supernatant was stored at −80 °C until use.

2.5. Total RNA extraction and reverse transcription (RT)

After 3 h incubation, pituitaries from each well were collected and rinsed in a 1.5 mL Eppendorf tube with 0.5 mL pre-cold TRI reagent (Sigma-Aldrich, USA), and then stored at −80 °C until use. Total RNA was extracted according to the manufacturer's instructions of TRI. The quality and concentration of RNA was measured at 260 nm with a purity ratio of A260/A280 on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc. USA). First-strand cDNA of each sample was synthesized from 0.5 μg total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied biosystems, USA) according to the user's manual.

Table 1
Primers used in the present study.

Gene	Sequence (5′–3′)	Accession number	Product size	Efficiency (%)
il1β	F: CTGAAGCCAGACCTGTAGCC R: GCAACCTCCTCTAGGTGCAG	AJ223954	100 bp	100.3
il8	F: CACAGACAGAGAAGGAAGGAAAG R: TGCTCATCTTGGGGTTACAGA	AJ279069	162 bp	97.1
tnfα1	F: AGCATGGAAGACCGTCAACGAT R: ACCCTCTAAATGGATGGCTGCTT	AJ277604	131 bp	111.8
ifnγ	F: GAAGGCTCTGTCCGAGTTCA R: TGTGTGATTTGAGCCTCTGG	AJ616215	119 bp	107.6
tgfβ1b	F: GGGCGACAGCAGACGATACC R: TTCAGCCATTCCTTGAGGGT	NM_001281366.1	102 bp	101.4
il10	F: CGACTTTAAATCTCCCATCGAC R: GCATTGGACGATCTCTTTCTT	AB118099.1	70 bp	127.2
mhcIIα	F: ACACCCTTATCTGCCACGTC R: TCTGGGGTGAAGCTCAGACT	AJ251432	160 bp	107.0
c3	F: GAGATGGCCTCCAAGAAGATAGAA R: ACCGCATGTACGCATCATCA	L24433.1	91 bp	103.6
mif	F: TAGGCTGTGGAGAGACCAGC R:GTCTGCATAAAGCAAGGGTT	NM_001124581	86 bp	109.2
crhbp	F: TGGGACGGTCAGGAGAAC R: GACTGTCAGCGTGAAGGA	NM_001124631.1	94 bp	109.1
pomca	F: TCCCAGCGAGATGAGACGA R: CTCCTGAAGACTAAACACCCC	NM_001124718.1	118 bp	74.2
pomcb	F: GATATTAGCATTGCCCTAGAT R: ATGGTCACTGTTACCGAAGA	NM_001124719.1	152 bp	91.3
gr1	F: CGCAGCAGAACCAACAGTTG R: ATGAGGGCGTCCAAGTACAGA	Z54210.1	107 bp	103.6
ef1α	F: CAAGGATATCCGTCGTGGCA R: ACAGCGAAACGACCAAGAGG	AF498320	327 bp	96.6

2.6. Reverse transcription quantitative real-time PCR (RT-qPCR)

In order to investigate the alteration of target genes at the transcription level, RT-qPCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) in a CFX Touch™ Real-Time PCR Detection System (Bio-Rad, USA). In brief, a volume of 10 μL containing 0.4 μM of each upstream and downstream primer (Table 1), 2 μL of cDNA product, and 5 μL of 2 × iTaq Universal SYBR green Supermix were used for the RT-qPCR reaction. The cycling condition consisted of an initial denaturation cycle for 5 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C. A default melting curve analysis was carried out after the completion of RT-qPCR to verify no non-specific amplification. Based on the available report (Attaya et al., 2018), the housekeeping gene *EF1A* was used as a reference for normalization. Standard curves were generated using 2.5^{-1}–2.5^{-7} dilution series of cDNA. The amplification efficiency and product size were listed in Table 1. Data were analyzed using a reported method (Pfaffl, 2001).

2.7. Statistics

Statistical analyses were performed with Prism software (GraphPad Software Inc., San Diego, CA) using either one-way ANOVA followed by Fisher's LSD post-hot test, or unpaired student's *t*-test if the equal variances were not assumed. Differences among groups were considered significant when $P < 0.05$. All results were expressed as mean ± SEM.

3. Results

3.1. Expression of pro-inflammatory cytokines

The expression of *il1β* in trout pituitary among different groups were significant different (F = 12.47, $P < 0.0001$). As expected, bacterin caused the significant up-regulation of *il1β* (Fig. 1A). After 3 h incubation with 1:50 diluted bacterin or with bacterin plus 50 ng/mL recombinant IL1β, the endogenous transcription of *il1β* in the rainbow trout pituitary was significantly increased ($P < 0.0001$), by 5.0 and 4.1 fold respectively. Nevertheless, supplementation of recombinant IL1β alone in the incubation medium caused non-significant alteration of *il1β* transcript. Incubation with the medium derived from the bacterin treated or control spleen led to the significant raise or decrease of *il1β* ($P < 0.01$). 100 nM CRH as well as 100 ng/L cortisol significantly suppressed the expression of *il1β* to 18.3% and 10.7%, respectively. Nonetheless, this gene was distinctly up-regulated in pituitaries simultaneously incubated by bacterin plus CRH or cortisol ($P < 0.05$).

Similarly than for *il1β*, the expression of *il8* in the trout pituitary of different groups were also significant different (F = 6.097, $P < 0.0001$). Bacterin caused a significant raise of *il1β*, by 5.33 folds, while medium from the bacterin treated spleen and bacterin plus CRH led to the up-regulation of *il1β* as well, by 2.23, 3.68 folds, respectively as well ($P < 0.05$) (Fig. 1B). Adding of IL1β alone did not lead to any alteration of *il8*, while the medium derived from the control spleen and CRH caused a slight decrease of this gene. Notably, the *il8* transcript was suppressed by cortisol, while this suppression was significantly rescued by simultaneously adding cortisol plus bacterin ($P < 0.01$) (Fig. 1B). The difference of *tnfα1* transcript abundance in the trout pituitary among different groups was not significant (F = 1.631, $P = 0.1334$). It was only induced in bacterin plus IL1β subgroup ($P < 0.01$), by 3.47 folds. All the other treatments just slightly altered the expression of this gene, however without significance (Fig. 1C). None of these treatments caused significant alteration of the *ifnγ* transcription in the pituitary of rainbow trout *in vitro* (F = 1.025, $P = 0.4343$) (Fig. 1D).

3.2. Expression of anti-inflammatory cytokines

The expression of *tgfβ1b* in trout pituitary among different groups were significant different (F = 5.492, $P < 0.0001$). The typical anti-inflammatory cytokine gene *tgfβ1b* was significantly suppressed by 3 h incubation with bacterin, both alone and plus with other stimulators such as IL1β, CRH and cortisol (Fig. 2A). Besides, the medium from the bacterin treated trout spleen caused evident decrease of *tgfβ1b* as well ($P < 0.01$). Administration of IL1β, CRH or cortisol alone caused a non-significant up-regulation of this gene (Fig. 2A). The expression of another anti-inflammatory gene *il10* was only significantly increased by

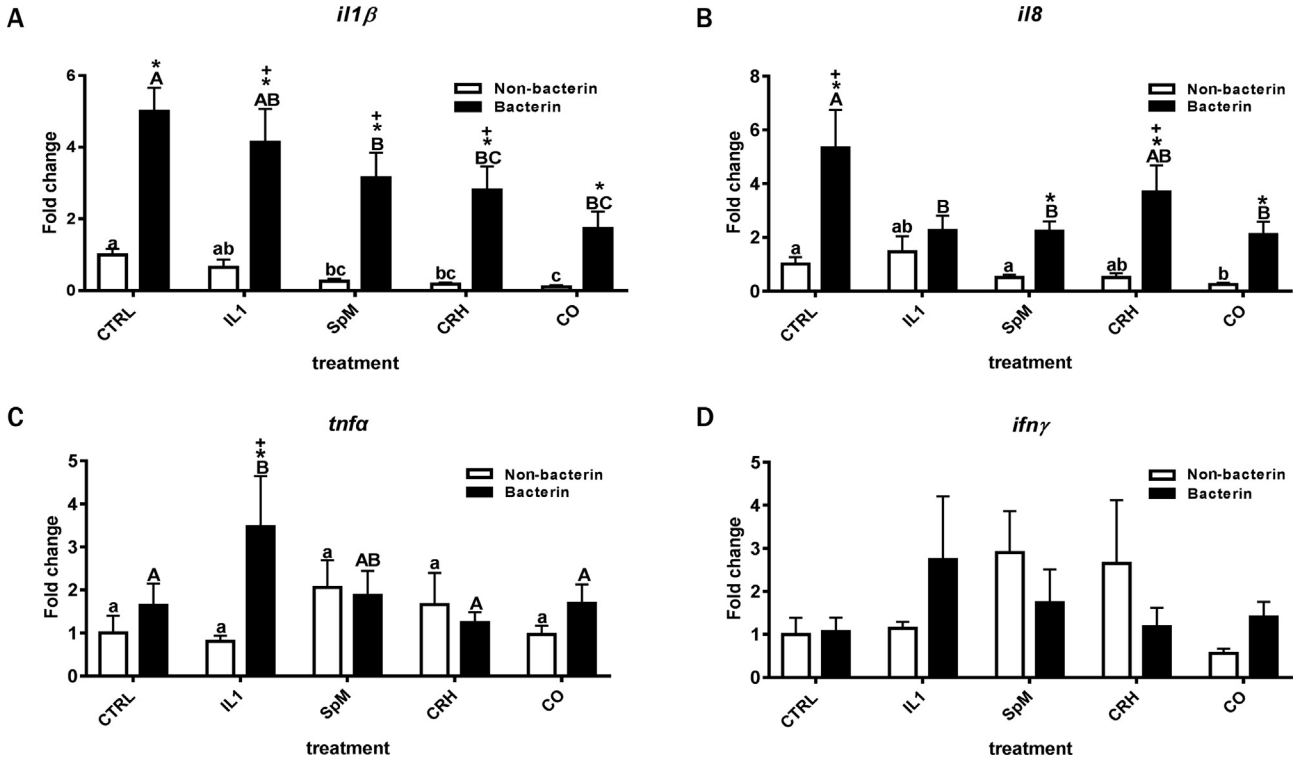

Fig. 1. Expression of pro-inflammatory cytokine genes *il1β* (A), *il8* (B), *tnfa1* (C), and *ifn*γ (D) in the pituitary of rainbow trout followed by 3 h incubation with various stimulatory agents. CTRL: control, pituitaries cultured with and without bacterin, respectively; IL1: pituitaries in both groups cultured with recombinant IL1β; SpM: pituitaries in non-bacterin and bacterin groups cultured with medium from *in vitro* cultured control and bacterin treated trout spleen, respectively; CRH: pituitaries in both groups cultured with CRH; CO: pituitaries in both groups cultured with cortisol. Different letters in lower case indicate the differences between pituitaries in non-bacterin group; different letters in upper case indicate the differences between pituitaries in bacterin group; * indicates the differences within the same subgroups between bacterin and non-bacterin groups; + indicates the differences of subgroups in bacterin group from the control of non-bacterin group.

incubation of bacterin alone ($P < 0.05$), while all the other treatments caused non-significant alteration of this gene (Fig. 2B).

3.3. Expression of genes encoding for some other soluble mediators of immunity

In order to assess whether the antigen process and presenting system and the complement system are also functional within the pituitary, the corresponding expression of *mhcIIα*, *c3* and *mif* which function as a immune regulators in all kinds of mammalian tissues, were also tested (Fig. 3). The expressions of *mhcIIα* ($F = 2.465$, $P = 0.0207$) and *mif* ($F = 2.647$, $P = 0.0137$) in trout pituitary among different groups were significant different, while that of *c3* was not ($F = 1.477$, $P = 0.1836$).

Incubation with IL1 and mediums from both control and bacterin treated trout spleen caused significant up-regulation of *mhcIIα* by 5.78, 4.17, 3.93 folds, respectively ($P < 0.05$), while other stimulatory agents caused non-significant alteration of this gene. Regarding *c3*, bacterin plus IL1, and mediums from both control and bacterin treated trout spleen led to up-regulation of this gene in trout pituitary, however, the significance was just observed in the SpM subgroup of non-bacterin group ($P < 0.05$). Down-regulation of *mif* was observed in

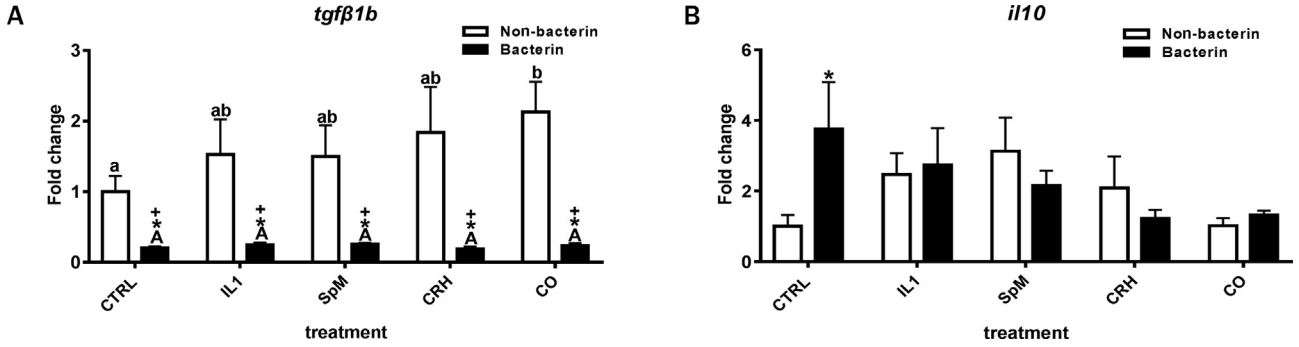

Fig. 2. Expression of anti-inflammatory cytokines genes *tgfβ1b* (A) and *il10* (B) in the pituitary of rainbow trout followed by 3 h incubation with various stimulatory agents. CTRL: control, pituitaries in non-bacterin and bacterin groups cultured with and without bacterin, respectively; IL1: pituitaries in both groups cultured with recombinant IL1β; SpM: pituitaries in non-bacterin and bacterin groups cultured with medium from *in vitro* cultured control and bacterin treated trout spleen, respectively; CRH: pituitaries in both groups cultured with CRH; CO: pituitaries in both groups cultured with cortisol. Different letters in lower case indicate the differences between pituitaries in non-bacterin group; different letters in upper case indicate the differences between pituitaries in bacterin group; * indicates the differences within the same subgroups between bacterin and non-bacterin groups; + indicates the differences of subgroups in bacterin group from the control of non-bacterin group.

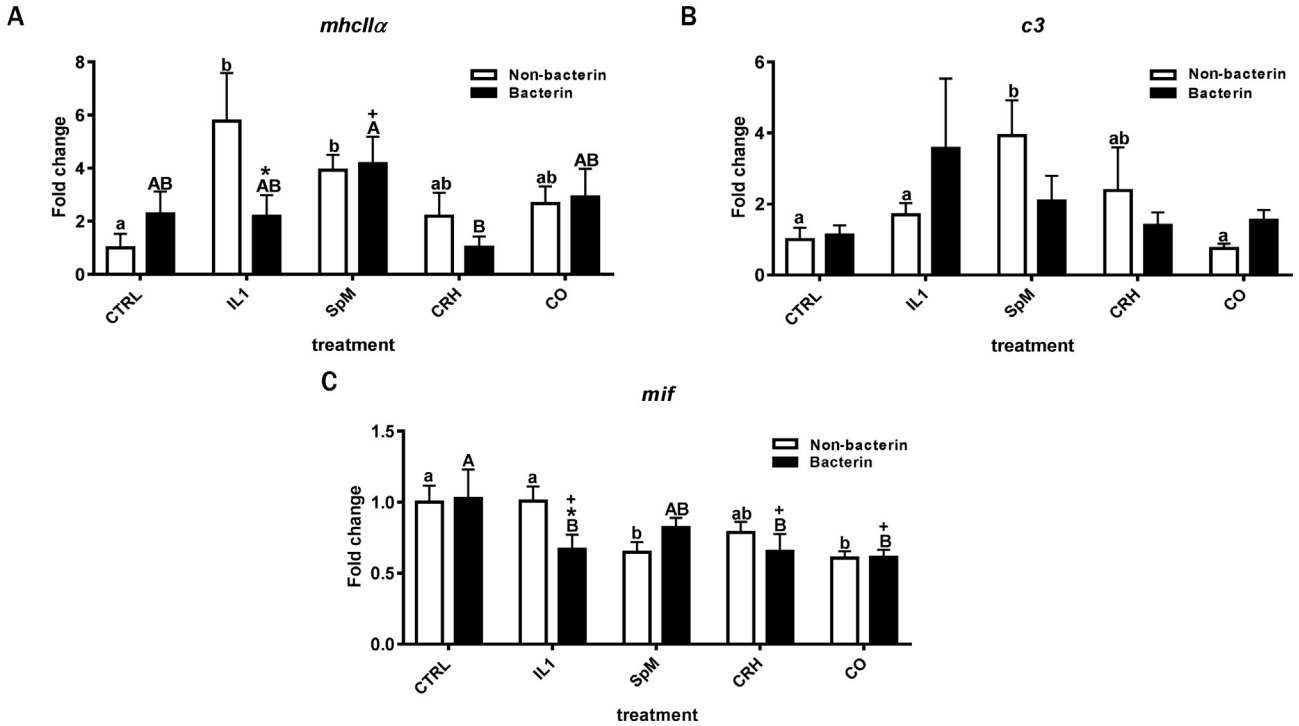

Fig. 3. Expression of *mhcIIα* (A), *c3* (B), and *mif* (C) genes in the pituitary of rainbow trout followed by 3 h incubation with various stimulatory agents. CTRL: control, pituitaries in non-bacterin and bacterin groups cultured with and without bacterin, respectively; IL1: pituitaries in both groups cultured with human recombinant IL1β; SpM: pituitaries in non-bacterin and bacterin groups cultured with medium from *in vitro* cultured control and bacterin treated trout spleen, respectively; CRH: pituitaries in both groups cultured with CRH; CO: pituitaries in both groups cultured with cortisol. Different letters in lower case indicate the differences between pituitaries in non-bacterin group; different letters in upper case indicate the differences between pituitaries in bacterin group; * indicates the differences within the same subgroups between bacterin and non-bacterin groups; + indicates the differences of subgroups in bacterin group from the control of non-bacterin group.

SpM and CO subgroups of non-bacterin group, while in IL1 and CO subgroups of the bacterin group ($P < 0.05$).

3.4. Expression of genes function in the HPA axis

With the aim to investigate the effects of immune stimulators on the local endocrine system of fish pituitary, the expression of *crhbp*, *pomca*, *pomcb* and *gr1*, which are encoding the binding protein of CRH, proopiomelanocortin-a/-b, and receptor of glucocorticoid, respectively, were assessed in the pituitary of rainbow trout after various stimulations (Fig. 4).

Expression of *crhbp* showed non-significant alteration following 3 h incubation with all kinds of stimulators ($F = 1.337$, $P = 0.2425$). mRNA of *pomca* displayed a decreasing trend with addition of these stimulators, and the decrease was significant in the CRH and CO subgroups of both non-bacterin and bacterin groups, as well as in the SpM subgroup of no-bacterin group ($F = 2.347$, $P = 0.027$). The expression of *pomcb* was similar with that of *crhbp*, while it was significantly increased after the incubation with IL1 + ($P < 0.05$); mRNA abundance of *gr1* was just significant altered in the BA group ($P < 0.05$), and showed almost no changes in other groups ($F = 1.228$, $P = 0.2998$).

4. Discussion

Fish live in an aquatic environment full of microorganism, including potential pathogens. If pathogens succeed in penetrating the epithelia, the pathogens will be recognized, and then provoke immune responses, including cytokine release, antigen presentation and bacteriolysis. The release of cytokines will unleash the nonspecific inflammatory process and the antigenic presentation will set the bases for further specific responses and memory (Castro and Tafalla, 2015). This is what will happen in immune organs and in mucosal-associated lymphoid tissues in response to pathogen attack. Pituitary is a small but pivotal endocrine organ that functions as the bridge between brain and the target tissues for the released hormones. Protected by the blood brain barrier, the pituitary is normally away from pathogens. However, results from mammals showed that some cytokines and antigen presenting molecules are stimulated in response to immune stimulators (Silverman et al., 2005). Whether a relevant immune response could be elicit in a fish pituitary was unknown. In the present study, the expression of *il1β*, *il8*, *tnfα1*, *ifnγ*, *tgfβ1b*, *il10*, *c3*, *mhcIIα* is similar to what has been observed in mammalian pituitary (Arzt, 2001; Utsuyama and Hirokawa, 2002; Francis et al., 2003, 2008), which suggested the ability of fish pituitary to elicit the pro- and anti-inflammatory response. The induction of *il1β*, *il8 tnfα* and *mhcIIα,* while suppression *of tgfβ1b* following immune stimulation further verified a functional immune response in trout pituitary.

Although the specific functionality of IL1β in fish pituitary has not been elucidated before, it has long been proposed to be functional in the mammalian pituitary. Specifically, IL1β was reportedly to stimulate the GH release from cultured ovine pituitary cells (Fry et al., 1998), and the induction of high mobility group 1 protein in pituicytes by IL1 was supposed to be the way to participate in the regulation of neuroendocrine and immune response (Wang et al, 1999). Moreover, the predominant effects of cytokines in the central nervous system were claimed to stimulate the HPA axis and to suppress the HPT (thyroid) and gonadal axes, and growth hormone (GH) release (Haddad et al, 2002). Although in the present work we could not get trout IL1β (Hong et al., 2001, Peddie et al., 2001) fish immune cells were able to recognize and respond to human IL-1 (Sigel et al, 1986; Hamby et al, 1986); the significant induction of mhcIIα in trout pituitary by recombinant human IL1β further confirmed the successful recognition and response. However, just a small alteration of crhbp and pomcb can be induced by IL1 in the present study, which suggested that exogenous IL1 has just a small effect on the endocrine response of fish pituitary. In the present study, bacterin induced significant raise of the endogenous

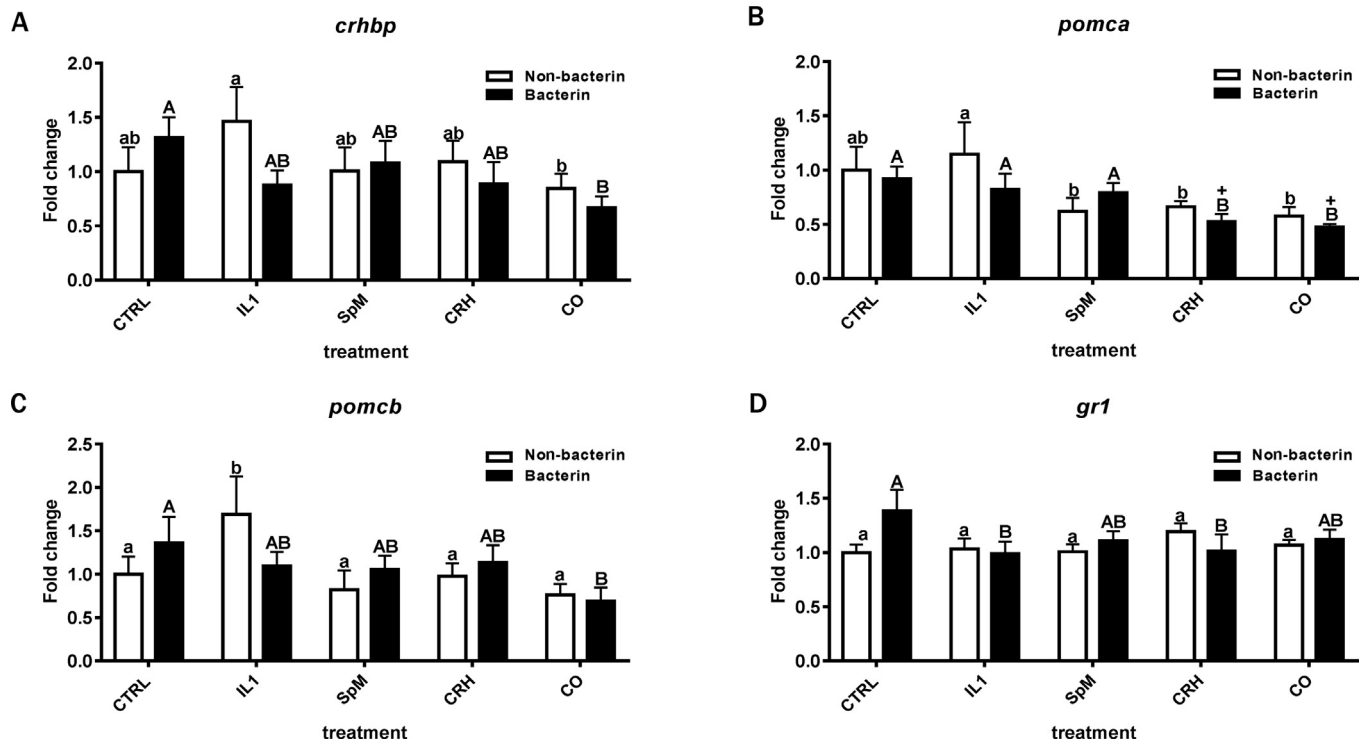

Fig. 4. Expression of *crhbp* (A), *pomca* (B), *pomcb* (C), and *gr1* (D) in the pituitary of rainbow trout followed by 3 h incubation with various stimulatory agents. CTRL: control, pituitaries in non-bacterin and bacterin groups cultured with and without bacterin, respectively; IL1: pituitaries in both groups cultured with recombinant IL1β; SpM: pituitaries in non-bacterin and bacterin groups cultured with medium from *in vitro* cultured control and bacterin treated trout spleen, respectively; CRH: pituitaries in both groups cultured with CRH; CO: pituitaries in both groups cultured with cortisol. Different letters in lower case indicate the differences between pituitaries in non-bacterin group; different letters in upper case indicate the differences between pituitaries in bacterin group; * indicates the differences within the same subgroups between bacterin and non-bacterin groups; + indicates the differences of subgroups in bacterin group from the control of non-bacterin group.

expression of *il1β* and *il8*, while these pro- or anti-inflammatory genes in trout pituitary were not induced by exogenous human IL1β, which suggested that the immune response between immune organs and pituitary might be different. This view was supported by the previous report that after peripheral administration of LPS, kinetics and dose-response curve of cytokine genes in the pituitary were different from those in spleen (Laye et al., 1994).

The antigen presentation by MHC is the basis for the adaptive immune response (Castro and Tafalla, 2015), and MHCII genes are constitutively expressed by professional antigen presenting cells. Detection of MHCII in the stellate cells of rat pituitary is a proof for the function of non-immune cells in the antigen presentation (Glennon et al., 2015). Although the stellate cell network of fish was reportedly to be different from that of mammals (Golan et al., 2016), the detection of *mhcIIa* gene and its induction following exposure to recombinant IL1β or medium from the cultured spleen suggested a constitutive expression of this gene, and therefore some potential antigen presenting cells could exist in fish pituitary. Among various regulators, IFN-γ is the most prominent cytokine to induce the expression of MHCII in mammals (Romieu-Mourez et al., 2007; Choi et al., 2011), and in some cell types, such as in synovial cells, MHCII can only be induced by IFNγ (Wicks et al., 1992). However, no significant alteration of *ifnγ* was observed here, which implies that the induction of *mhcIIα* in trout pituitary after exposure to IL1β or medium from *in vitro* cultured trout spleen might take place through a non-IFNγ pathway.

It is interesting to note that the direct bacterin exposure caused an important fold raise of pro-inflammatory cytokines though non-significant alteration of *mhcIIα*, however the opposite results were obtained after treatment with exogenous IL1β alone or with spleen derived medium. Fish spleen is a key immune organ, full of macrophages, lymphocytes and melanomacrophages, and it is an important site for cytokine production (Castro and Tafalla, 2015). Normally, protected by the blood brain barrier, pathogens can hardly access to the brain or pituitary, however, some mediators such as cytokines can be available through the blood (Banks et al., 1995). Based on this, we supposed that after experimental infection, the pituitary can get information from the cytokines or from some other mediators associated to immune organs promoting local cytokine production or antigen process and presentation activity. This would provide the ability and memory to get rid of the pathogens that might eventually reach the pituitary. However, down-regulation of the *il1β* and *il8* in the trout pituitary incubated with medium from the normal spleen also suggested the possibility that some anti-immune mediators might be produced by the immune organ, and they could be functional to potentially suppress the immune activities in the pituitary.

Altogether, in the present study, the expression of genes encoding for cytokines, complement factors, antigen-processing factors and the up-regulation of *il1β*, *il8* and *mhcIIa* in trout pituitary following bacterin stimulation seem to suggest that, similar to mammals, the fish pituitary can have immune functions as to elicit both innate and adaptive immune responses. This was indicated by the cytokine production and the antigen presentation cells activity recorded within the pituitary of rainbow trout.

CRH is a canonical corticotropin that promotes the secretion of ACTH. It has been proposed in mammals that it modulates the immune responses via two pathways: brain-related CRH acting as anti-inflammatory via stimulation of glucocorticoid and catecholamine, and peripheral CRH acting as pro-inflammatory through direct action on immune cells (Karalis et al., 1997; Quintanar and Guzman-Soto, 2013). Thus, the effects of CRH on the expression of cytokines such as IL1, IL2, IL6, IL8, TNFα in immune cells including mast cells, T and B lymphocytes is predominantly pro-inflammatory (Crompton et al., 2003; Singh and Leu, 1990). However, it can inhibit LPS-mediated IL1 and IL6 production in peripheral blood monocytes and mature neutrophils

(Hagan et al., 1992; Radulovic and Spiess, 2001). In the present study, we observed a comparable anti-inflammatory effect of CRH or cortisol, not just on the inhibition of pro-inflammatory cytokines *il1β*, *il8* and *mhcIIα*, but also on the enhancement of the anti-inflammatory cytokine gene *tgfβ1b*. The results strongly suggest that, besides via cortisol, another loop between neuroendocrine system and the immune system might be present in fish through the direct action of CRH, and that exogenous CRH plays a role as a direct actor other than the peripheral effect driven through the HPI axis. Such an increasingly complex role of CRH in peripheral tissues is supported by mammalian studies in which CRH has been shown to have specific actions in tissues such as gut motility and mucosal alterations after stress episodes (Tache and Perdue, 2004).

In mammals, HPA axis regulates the immune response, not just through cortisol, but also via CRH and ACTH directly on the immune cells, since these stress hormones and their receptors have been characterized in immune cells (Procaccini et al., 2014). On the other hand, it is now clear that genes related to immune responses are expressed in the pituitary, and that exogenous cytokines can play a role in the local immune response of pituitary in both mammals and fish. However, regarding the effects of immune stimuli on the pituitary stress response, the real effects are still under debate. Early results supported that it was unlikely for a cytokine like IL1 to produce effects directly on the rat pituitary gland (Uehara et al., 1987), while some latter data support the view that cytokines incubation for more than 4 h can directly stimulate hormone release from the pituitary gland (Silverman et al., 2005). In the present study, notable alterations of some immune-related genes were observed in rainbow trout pituitary following bacterin, IL1β, medium from the bacterin-treated spleen, CRH, or cortisol, whereas just slight changes of genes involved in the endocrine system were observed after the same treatments. This reduced response might be due to insufficient exposure time or less effects on the transcription and synthesis of hormones in pituitary in response to immune modulators. Although pro-inflammatory cytokines such as IL1 and TNF induce the expression of POMC gene, it was reported that POMC was Nur77 induction and activation dependent (Kovalovsky et al., 2004), thus the unchanged expression of *pomca*, *pomcb* and *crhbp* in the pituitary after incubation with CRH or other stimulators might be associated to the lack of some endogenous co-factors as well. Further work is being carried out to test these hypotheses *in vivo*.

5. Conclusion

The constitutive expression and alteration of some pro- and anti-inflammatory genes including *il1β*, *il8*, *tnfα1*, *tgfβ1b*, the complement component *c3* gene, and the antigen presentation *mhcIIα* gene suggests that a relevant local immune activity is present in the pituitary of rainbow trout. Thus, fish pituitary is able to elicit both innate and adaptive immune responses, though with differences depending on whether the pathway is direct or mediated by peripheral tissue activity. Moreover, immune modulators reaching the pituitary from other tissues with a relevant immune function such as the spleen might play also a role as regulators of the local immune fish pituitary response. On the other side, the slight alteration observed in the HPI axis stress genes suggests a low relevance of immune stimuli on the endocrine stress response system of the fish pituitary, at least regarding the short-term acute response at the *in vitro* level.

Acknowledgements

Thanks are given to Joan Baro for the supply of trout. This research was funded by Ministry of Science, Innovation and Universities, grants AGL2016-76069-C2- 2-R and AGL2016-81808-REDT of MICINN (Spain) with the support of FEDER funds (European Union). XL was recipient of a grant named the *Major programme of science and technology commission foundation of Chongqing* (cstc2014yykfc80001) from the Key Laboratory of Freshwater Fish Reproduction and Development (Ministry of Education), School of Life Sciences, Southwest University, Chongqing, China. LT is a member of the Xarxa d'Aquicultura de Catalunya.

References

Arzt, E., 2001. gp130 cytokine signaling in the pituitary gland: a paradigm for cytokine-neuro-endocrine pathways. J. Clin. Invest. 108, 1729–1733. https://doi.org/10.1172/JCI14660.

Attaya, A., Wang, T., Zou, J., et al., 2018. Gene expression analysis of isolated salmonid GALT leucocytes in response to PAMPs and recombinant cytokines. Fish Shellfish Immunol. 80, 426–436. https://doi.org/10.1016/j.fsi.2018.06.022.

Banks, W.A., Kastin, A.J., Broadwell, R.D., 1995. Passage of cytokines across the blood-brain barrier. Neuroimmunomodulat. 2, 241–248. https://doi.org/10.1159/000097202.

Bellavance, M.A., Rivest, S., 2014. The HPA – immune axis and the immunomodulatory actions of glucocorticoids in the brain. Front. Immunol. 5, 136. https://doi.org/10.3389/fimmu.2014.00136.

Blalock, J.E., Weigent, D.A., 1994. Pituitary control of immune cells. Immunol. Today 15, 39. https://doi.org/10.1016/0167-5699(94)90024-8.

Castro, R., Tafalla, C., 2015. Overview of fish immunity. In: Beck, B.H., Peatman, E. (Eds.), Mucosal health in aquaculture. Academic Press, Elsevier Inc., pp. 3–54.

Choi, N.M., Majumder, P., Boss, J.M., 2011. Regulation of major histocompatibility complex class II genes. Curr. Opin. Immunol. 23, 81–87. https://doi.org/10.1016/j.coi.2010.09.007.

Crompton, R., Clifton, V.L., Bisits, A.T., et al., 2003. Corticotropin-releasing hormone causes vasodilation in human skin via mast cell-dependent pathways. J. Clin. Endocrinol. Metab. 88, 5427–5432. https://doi.org/10.1210/jc.2003-030377.

Dunn, A.J., 2007. The HPA axis and the immune system: a perspective. Neuroimmune Bio. 7, 3–15. https://doi.org/10.1016/S1567-7443(07)00201-3.

Francis, K., Lewis, B.M., Akatsu, H., et al., 2003. Complement C3a receptors in the pituitary gland: a novel pathway by which an innate immune molecule releases hormones involved in the control of inflammation. FASEB J. 17, 2266–2268. https://doi.org/10.1096/fj.02-1103fje.

Francis, K., Lewis, B.M., Monk, P.N., et al., 2008. Complement C5a receptors in the pituitary gland: expression and function. J. Endocrinol. 199, 417–424. https://doi.org/10.1677/JOE-08-0110.

Fry, C.L., Gunter, D.R., McMahon, C.D., et al., 1998. Cytokine-mediated growth hormone release from cultured ovine pituitary cells. In: Neuroendocrinology (Basel) 68. pp. 192–200. https://doi.org/10.1159/000054366.

Glennon, E., Kaunzner, U.W., Gagnidze, K., et al., 2015. Pituitary dendritic cells communicate immune pathogenic signals. Brain Behav. Immun. 50, 232–240. https://doi.org/10.1016/j.bbi.2015.07.007.

Golan, M., Hollander-Cohen, L., Levavi-Sivan, B., 2016. Stellate cell networks in the teleost pituitary. Sci. Rep. 6 (24426), 2016. https://doi.org/10.1038/srep24426.

Haddad, J.J., Saade, N.E., Safieh-Garabedian, B.S., et al., 2002. Cytokines and neuro–immune–endocrine interactions: a role for the hypothalamic–pituitary–adrenal revolving axis. J Neuroimmunol. 133, 1–19. https://doi.org/10.1016/s0165-5728(02)00357-0.

Hagan, P., Poole, S., Bristow, A.F., 1992. Immunosuppressive activity of corticotrophin-releasing factor. Inhibition of interleukin-1 and interleukin-6 production by human mononuclear cells. Biochem. J. 281, 251–254. https://doi.org/10.1042/bj2810251.

Hamby, B.A., Huggins Jr., E.M., Lachman, L.B., et al., 1986. Fish lymphocytes respond to human IL-1. Lymphokine Res. 5, 157–162. https://doi.org/10.1016/0165-5728(86)90077-9.

Hanisch, A., Dieterich, K.D., Dietzmann, K., et al., 2000. Expression of members of the interleukin-6 family of cytokines and their receptors in human pituitary and pituitary adenomas. J. Clin. Endocrinol. Metab. 85, 4411–4414. https://doi.org/10.1210/jcem.85.11.7122.

Hodkinson, C.F., Simpson, E.E., Beattie, J.H., et al., 2009. Preliminary evidence of immune function modulation by thyroid hormones in healthy men and women aged 55–70 years. J. Endocrinol. 202, 55–63. https://doi.org/10.1677/JOE-08-0488.

Hong, S., Zou, J., Crampe, M., et al., 2001. The production and bioactivity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1 beta. Vet. Immunol. Immunopathol. 81, 1–14. https://doi.org/10.1016/S0165-2427(01)00328-2.

Karalis, K., Muglia, L.J., Bae, D., et al., 1997. CRH and the immune system. J. Neuroimmunol 72, 131–136. https://doi.org/10.1016/S0165-5728(96)00178-6.

Katoh, K., Asari, M., Ishiwata, H., et al., 2004. Saturated fatty acids suppress adrenocorticotropic hormone (ACTH) release from rat anterior pituitary cells *in vitro*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 137, 357–364. https://doi.org/10.1016/j.cbpb.2003.10.011.

Kovalovsky, D., Paez Pereda, M., Labeur, M., et al., 2004. Nur77 induction and activation are necessary for interleukin-1 stimulation of proopiomelanocortin in AtT-20 corticotrophs. FEBS Lett. 563, 229–233. https://doi.org/10.1016/S0014-5793(04)00303-5.

Laye, S., Parmet, P., Goujon, E., et al., 1994. Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice. Mol. Brain Res. 27, 157–162. https://doi.org/10.1016/0169-328X(94)90197-X.

Metz, J.R., Huising, M.O., Leon, K., et al., 2006. Central and peripheral interleukin-1beta and interleukin-1 receptor I expression and their role in the acute stress response of common carp, *Cyprinus carpio* L. J. Endocrinol. 191, 25–35. https://doi.org/10.1677/joe.1.06640.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids Res. 29, e45. https://doi.org/10.1093/nar/29.9.e45.

Peddie, S., Zou, J., Cunningham, C., Secombes, C.J., 2001. Rainbow trout (*Oncorhynchus mykiss)* recombinant IL-1β and derived peptides induce migration of head-kidney leucocytes *in vitro*. Fish Shellfish Immunol. 11, 697–709. https://doi.org/10.1006/fsim.2001.0348.

Procaccini, C., Pucino, V., De Rosa, V., et al., 2014. Neuro-endocrine networks controlling immune system in health and disease. Front. Immunol. 5, 143. https://doi.org/10.3389/fimmu.2014.00143.

Quintanar, J.L., Guzman-Soto, I., 2013. Hypothalamic neurohormones and immune responses. Front. Integr. Neurosci. 7, 56. https://doi.org/10.3389/fnint.2013.00056.

Radulovic, M., Spiess, J., 2001. Immunomodulatory role of the corticotropin-releasing factor. Arch. Immunol. Et. Ther. Exp. 49, 33–38.

Romieu-Mourez, R., Francois, M., Boivin, M. N., et al. 2007. Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-gamma, TGF-.

Sigel, M.M., Hamby, B.A., Huggins Jr., E.M., 1986. Phylogenetic studies on lymphokines. Fish lymphocytes respond to human IL-1 and epithelial cells produce an IL-1 like factor. Vet. Immunol. Immunopathol. 12, 47–58. https://doi.org/10.1016/0165-2427(86)90109-1.

Silverman, M.N., Pearce, B.D., Biron, C.A., et al., 2005. Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. Viral Immunol. 18, 41–78. https://doi.org/10.1089/vim.2005.18.41.

Singh, V.K., Leu, S.J., 1990. Enhancing effect of corticotropin-releasing neurohormone on the production of interleukin-1 and interleukin-2. Neurosci. Lett. 120, 151–154. https://doi.org/10.1016/0304-3940(90)90025-5.

Tache, Y., Perdue, M.H., 2004. Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. Neurogastroenterol. Motil. 16, 137–142. https://doi.org/10.1111/j.1743-3150.2004.00490.x.

Uehara, A., Gottschall, P.E., Dahl, R.R., et al., 1987. Interleukin-1 stimulates ACTH release by an indirect action which requires endogenous corticotropin releasing-factor. Endocrinology 121, 1580–1582. https://doi.org/10.1210/endo-121-4-1580.

Utsuyama, M., Hirokawa, K., 2002. Differential expression of various cytokine receptors in the brain after stimulation with LPS in young and old mice. Exp. Gerontol. 37, 411–420. https://doi.org/10.1016/S0531-5565(01)00208-X.

Wang, H.C., Vishnubhakat, J.M., Bloom, O., et al., 1999. Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. Surgery 126, 389–392. https://doi.org/10.1016/S0039-6060(99)70182-0.

Wicks, I.P., Leizer, T., Wawryk, S.O., et al., 1992. The effect of cytokines on the expression of MHC antigens and ICAM-1 by normal and transformed synoviocytes. Autoimmunity 12, 13–19. https://doi.org/10.3109/08916939209146125.