

Corticotropin-releasing hormone-receptor 1 (CRH-R1) and CRH-binding protein (CRH-BP) are expressed in the gills and skin of common carp *Cyprinus carpio* L. and respond to acute stress and infection

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Summary

We established that corticotropin-releasing hormone (CRH), CRH-binding protein (CRH-BP) and CRH-receptor 1 (CRH-R1) are expressed in the gills and skin of common carp *Cyprinus carpio*, an early vertebrate. Immunoreactive CRH was detected in macrophage-like cells in gills and skin, in fibroblasts in the skin and in endothelial cells in the gills. The involvement of the CRH system in gills and skin was investigated in response to infection and in an acute restraint stress paradigm. Carp were infected with the protozoan leech-transmitted blood flagellate *Trypanoplasma borreli* and subjected to acute restraint stress by netting for 24 h. The expression of CRH-BP and CRH-R1 genes in the gills and in the skin is

downregulated after both infection and restraint. Thus the peripheral CRH system reacts to infection and stress. The gills and skin separate the internal from the external environment and are permanently exposed to stress and pathogens. Because of their pivotal role in maintaining the homeostatic equilibrium, these organs must act locally to respond to diverse stresses. Clearly, the CRH system is involved in the response of the integument to diverse stresses at the vulnerable interface of the internal and external milieu.

Key words: fish, corticotropin-releasing hormone, cRH-BP, cRH-R1, *Cyprinus carpio*, gills, skin.

Introduction

In fish, in analogy with mammals, the stress response consists of the activation of the sympathetic nervous system, as well as the hypothalamus–pituitary–interrenal axis (HPI). In response to hypothalamic release of corticotropin-releasing hormone (CRH) the pituitary gland increases the synthesis and release of pro-opiomelanocortin (POMC)-derived peptides (Wendelaar Bonga, 1997; Aguilera, 1998; Dautzenberg and Hauger, 2002). CRH exerts its effects *via* specific membrane receptors (Aguilera et al., 2001). At least two CRH receptors exist and they differ in their pharmacological properties and tissue distribution.

CRH expression has been detected in a plethora of mammalian organs including skin, endometrium, placenta, uterus, ovary, testis, spleen, pancreas, liver, stomach, small and large intestine, adrenal and thyroid gland. Furthermore, CRH is produced by various immune cells, including macrophages (Baker et al., 2003) as a proinflammatory agent (Karalis et al., 1997), which is illustrated by the increased CRH expression during experimentally induced inflammation (Hargreaves et al., 1989) or in chronic inflammatory diseases, such as rheumatoid arthritis (Crofford et al., 1992, 1993).

CRH receptors (CRH-R) have been identified in the skin, adrenal gland, testis, ovary, prostate, kidney, liver, gut, spleen, circulating immune cells, synovium, heart, skeletal muscle, uterine myometrium, vascular endothelium, arterial smooth muscle, endometrium, placenta (reviewed by Slominski and Wortsman, 2000), arterioles, lungs and intestine (Coste et al., 2002). CRH-R1 is known to be present in the pituitary gland, brain and splenic neutrophils, granulocytes, and CRH-R2 is present in the heart, discrete areas of the brain (Chen et al., 1993; Lovenberg et al., 1995; Perrin et al., 1995; Radulovic et al., 1999; Coste et al., 2002), skeletal muscle, arterioles, lungs and intestine (Coste et al., 2002).

The bioactivity of CRH (and related peptides) depends on CRH-binding protein (CRH-BP), which determines the concentration of bioavailable CRH and may influence peptide bioactivity and half-life (Potter et al., 1991; Seasholtz et al., 2002). As such CRH-BP may act as a carrier protein that prevents CRH degradation and facilitates the delivery of peptides to distant sites (Seasholtz et al., 2002). The colocalisation of CRH-BP and CRH in both the rostral pars distalis as well as in the pars intermedia of carp (Huising et al.,

2004) substantiates that CRH-BP is also a regulator of CRH in the pituitary gland of fish.

Many of the effects of peripheral CRH appear to be related to stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Rothwell, 1994; Tsagarakis and Grossman, 1994; Heinrichs et al., 1995; Rivest and Rivier, 1995; Karalis et al., 1997; Webster et al., 1997; Turnbull and Rivier, 1999; Baigent, 2001). Acute immobilisation stress triggers CRH-mediated skin mast cell degranulation, an action that also involves neurotensin and substance P (Singh et al., 1999), and leads to vasodilation and increased vascular permeability (Theoharides et al., 1998). Furthermore, CRH induces the proliferation of keratinocytes *via* interaction with CRH receptors (Mitsuma et al., 2001).

Expression of CRH and CRH receptors in human skin has been documented (Pisarchik and Slominski, 2001, 2004). CRH-R1 mRNA expression was observed in keratinocytes, melanocytes, dermal fibroblasts (Slominski et al., 2004) and circulating immune cells (Slominski et al., 2001). Moreover, this expression is regulated by immune cytokines, UV radiation and skin pathologies (Slominski et al., 2001), factors that are associated with local damage.

Skin and gills in fish are directly and permanently exposed to the environment and thus to multiple physical, chemical and biological influences. A direct, local response of skin and gills to pathogens and chemical and physical stress is envisaged as an important means to guarantee internal homeostasis.

The fish gill is characterized by an extensive and delicate epithelium that separates the water from the blood. It is a physiologically diversified organ that serves respiration, osmoregulation, nitrogen excretion and acid–base balance, which are key processes that are strongly interrelated. The gill is the only organ that is perfused by the entire cardiac output and has an extensive vascular surface area in contact with the plasma (Olson, 1998); the gills play a significant, and in some instances dominant, role in endocrine regulation as an endocrine target as well as metabolically active tissue (Evans et al., 2005).

The skin protects the fish against injury and infection and is protected by a chemically and functionally complex mucus coat that is discharged by mucus cells in the epidermis. It contains a variety of biologically active compounds including peroxidase (Iger et al., 1994, 1995; Brokken et al., 1998), lysozyme (Rainger and Rowley, 1993), immunoglobulins, complement and C-reactive protein (Shephard, 1994).

CRH-BP, CRH-R1 and CRH have been identified in several species of fish, including carp, in which these proteins have been shown to be involved in the regulation of the acute stress response (Huising et al., 2004). To date, information on peripheral expression of these factors in fish is limited.

Given the singular importance of fish gills and skin, we investigated the presence of a local CRH system in these organs. To that end we assessed the expression of CRH, CRH-BP and CRH-R1 and their messengers by immunohistochemistry and real-time quantitative PCR in the gill and skin of carp under normal, stressful and pathological conditions.

Materials and methods

Common carp *Cyprinus carpio* L. were reared at 23°C in recirculating UV-treated tapwater at the ‘De Haar Vissen’ facility in Wageningen, The Netherlands. Fish were fed with pelleted dry food (Provimi, Rotterdam, The Netherlands) at a daily ration of 0.7% of their estimated body mass. R3×R8 carp are the offspring of a cross between fish of Polish origin (R3) and Hungarian origin (R8) (Irnazarow, 1995). The fish used in infection experiments were housed in a quarantine unit at Wageningen University. The restraint experiment was carried out in a purpose-built setup at the Radboud University, Nijmegen, The Netherlands. At the end of both experiments, fish were rapidly and irreversibly anaesthetised in the experimental tanks without prior handling with 0.2 g l⁻¹ tricaine methane sulphonate (TMS) buffered with 0.4 g l⁻¹ NaHCO₃ or with 0.1% 2-phenoxyethanol.

Infection with Trypanoplasma borreli

Three weeks before experiments were started, carp (*N*=14) were transferred to a quarantine unit and kept in a single experimental tank. The R3×R8 carp line is trypanotolerant (Saeij et al., 2003a). After 3 weeks (*t*=0) one group (*N*=8) was injected intramuscularly at the base of the dorsal fin with 10 000 *Trypanoplasma borreli* in 100 µl RPMI and was designated the infected group. The control group (*N*=6) was injected with 100 µl RPMI and was marked by a small fin clip. Three weeks post-infection, when parasitaemia reached peak values (Saeij et al., 2003b), the fish were irreversibly anaesthetised. Blood samples were collected for the determination of haematocrit, leucocrit and parasitaemia.

Restraint period

Two groups of fish (*N*=8) were housed in identical tanks and after 3 weeks (*t*=0) one group was restrained for 24 h, by netting. The other group did not receive any treatment (controls). Following this 24-h restraint period, both control and stressed fish were irreversibly anaesthetised. Blood samples were collected for the determination of haematocrit values as well as several plasma parameters.

Isolation of head kidney and gill phagocytes

Fish were anaesthetised and blood was collected by puncture of the caudal vessels. Head kidney and gill phagocytes were isolated as previously described (Verberg-van Kemenade et al., 1994). Samples of gill and head kidney were passed through a 50 µm nylon mesh using the barrel from a 10 ml syringe and suspended in RPMI 1640 medium adjusted to carp osmolarity containing 0.2% heparin (Leo Pharmaceutical Products, Weesp, The Netherlands). Cell suspensions were enriched for phagocytes on a 1.07 g cm⁻³ Percoll gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The phagocyte-enriched fraction from the 1.07 Percoll interface was collected and washed twice with RPMI medium. Viability was assessed by Trypan Blue exclusion. For RNA isolation 1×10⁷ cells were pelleted and the RNA was isolated using a RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the

manufacturer's instructions. For immunohistochemistry the pelleted cells were fixed in Bouin's solution.

Analysis of CRH, CRH-BP and CRH-R1 gene expression by RQ-PCR

RNA isolation and first strand cDNA synthesis

Gill and skin samples from carp at the end of both infection and restraint experiments were flash-frozen in liquid nitrogen and stored at -80°C . RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Single strand cDNA was constructed using Invitrogen reagents, according to the manufacturer's protocol. Briefly $1\ \mu\text{l}$ $10\times$ DNase I reaction buffer and $1\ \mu\text{l}$ DNase I were added to $1\ \mu\text{g}$ total RNA and incubated for 15 min at room temperature in a total volume of $10\ \mu\text{l}$. DNase I was inactivated by adding $1\ \mu\text{l}$ $25\ \text{mmol l}^{-1}$ EDTA and incubated at 65°C for 10 min. To each sample, 300 ng random hexamers, $1\ \mu\text{l}$ $10\ \text{mmol l}^{-1}$ dNTP mix, $4\ \mu\text{l}$ $5\times$ first Strand buffer, $2\ \mu\text{l}$ $0.1\ \text{mol l}^{-1}$ dithiothreitol and 10 U RNase inhibitor were added and the mixture was incubated for 10 min at room temperature and an additional 2 min at 37°C . Then, 200 U Superscript RNase H reverse transcriptase (RT) was added and the reactions were incubated for 50 min at 37°C . A non-RT control was included for each sample; cDNA was stored at -20°C .

Real time quantitative PCR

The primers for real time quantitative PCR (RQ-PCR) used in this study were designed and previously used by Huising et al. (2004). For RQ-PCR $5\ \mu\text{l}$ cDNA and forward and reverse primers ($300\ \text{nmol l}^{-1}$ each) were added to $12.5\ \mu\text{l}$ Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and made up with demineralised water to a volume of $25\ \mu\text{l}$. RQ-PCR (2 min 48°C , 10 min 95°C , 40 cycles of 15 s 95°C and 1 min 60°C) was carried out on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Data were analysed with the $\Delta\Delta\text{Ct}$ method. Dual internal standards (40S and β -actin) were incorporated in all RQ-PCR experiments and results were confirmed to be very similar following standardisation to either gene. The amplification efficiencies of all primer sets in this study are very similar and deviate no more than 5% of the optimal amplification value of 2.0. Only the results standardised for 40S expression are shown.

Immunohistochemistry

Samples of gill and skin, and the phagocytes isolated from gills and head kidney were fixed in Bouin's solution, dehydrated in a graded ethanol series (70, 80, 90, 95 and 100%), embedded in paraffin, cut into $5\ \mu\text{m}$ sections and mounted on PolysineTM-coated slides (Menzel-Glazer[®], Braunschweig, Germany).

The histological sections were cleared in Xylol and placed in a solution of 1% H_2O_2 in methanol (30 min) to remove endogenous peroxidase activity, rehydrated and washed in phosphate-buffered saline-Triton X-100 (PBST, 0.1% Triton X-100, pH 7.4) twice (5 and 15 min, respectively). The sections were incubated (1 h) in a humid chamber at room

temperature with 10% normal goat serum in PBST. CRH was detected with a rabbit anti-ovine CRH (1-41) antiserum (1:100; Biotrend, Cologne, Germany). CRH-BP was detected with a rabbit anti-human CRH-BP antiserum (hR CRH-BP 254-299; a generous gift from Prof Dr W. Vale (Potter et al., 1992) at a dilution of 1:1000.

Sections were incubated with primary antibodies overnight at 4°C (CRH-BP) or room temperature (CRH). Goat anti-rabbit IgG-biotin (1:200 Vector Laboratories, Burlingame, CA, USA) was used as second antibody followed by amplification using the Vectastain[®] ABC Amplification Kit (Vector Laboratories) according to the manufacturer's protocol. AEC (3-amino-6-ethylcarbazole; Sigma, St Louis, MO, USA) was used as a substrate. Controls for cross-reactivity of the secondary antibodies and for endogenous enzyme activity were included in all experiments and were negative. Nuclei were counterstained with Haematoxylin. After pre-absorption of the primary antibodies the target cells were negative or in some cases only slightly positive.

Blood analysis

Blood samples were spun down in a cooled (4°C) microcentrifuge (10 min at $9500\ \text{g}$, IEC micromax RF; Waldham, MA, USA) and the plasma was collected and stored at -20°C until use. Cortisol was measured by radioimmunoassay (RIA) as described previously (Huising et al., 2004). Plasma levels of Na^+ and K^+ , glucose, lactate, and the pH were determined with a Stat Profile[®]pHOx[®]Plus (Nova Biomedical, Waldham, MA, USA, USA).

Cell counting

CRH-BP-positive cells in the gill filaments from the restraint experiment were quantified in 10 views of the gill filament area using a stereological overlay method in which a grid was used to estimate the tissue area (in mm^2) (Mazon et al., 2004) and the results were expressed as number of cells mm^{-2} .

Statistical analysis

All statistical analyses were carried out with Graphpad Prism software (3.0). Differences were evaluated with a Student's *t*-test, $P < 0.05$ was taken as fiducial limit.

Results

CRH, CRH-BP and CRH-R1 expression at mRNA and protein level

The expression of CRH, CRH-R1 and CRH-BP was studied in the gills and skin of carp. Expression was plotted relative to the expression of ribosomal protein 40S. CRH expression was significant but very low in both skin and gills; CRH-R1 and CRH-BP were expressed more abundantly in both gills and skin (Fig. 1).

Immunohistochemical studies allowed us to visualise where CRH and CRH-BP proteins were expressed in the gills and skin. In the gills, macrophage-like cells, judged by their relatively large volume of cytoplasm and eccentric nucleus,

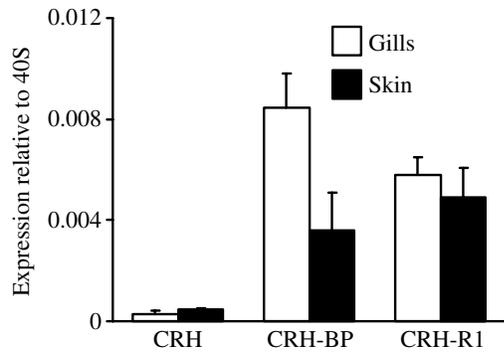


Fig. 1. Basal expression of CRH, CRH-R1 and CRH-BP in the gills and skin of *C. carpio*. Expression was assessed by RQ-PCR and is plotted relative to the expression of ribosomal 40S. Values are means \pm s.d. ($N=5$).

were positive for CRH or CRH-BP (Fig. 2A,B). Many cells of similar appearance were not immunoreactive to either CRH or CRH-BP antiserum. Other cells that are located within the basal layer in close proximity to the blood vessel were CRH-positive (Fig. 2A). CRH-BP-positive macrophage-like cells were seen mainly in the interface between the central venous sinus and the basal layer of the filament epithelia.

In the skin some of the CRH- and CRH-BP-positive cells had a similar macrophage-like appearance. CRH-positive cells were observed in the basal layer of the epidermis, close to the melanocytes and also in the dermis (Fig. 3A) and in the fibroblasts of the dermis (Fig. 3B). CRH-BP-positive cells were observed only in the dermis (Fig. 3C,D).

CRH- and CRH-BP-positive cells were also observed in the phagocytic fraction from head kidney (not shown) and gills (Fig. 4A,B). Expression of CRH, CRH-BP and CRH-R1 was always detectable in the isolated gill phagocytes (Fig. 4C).

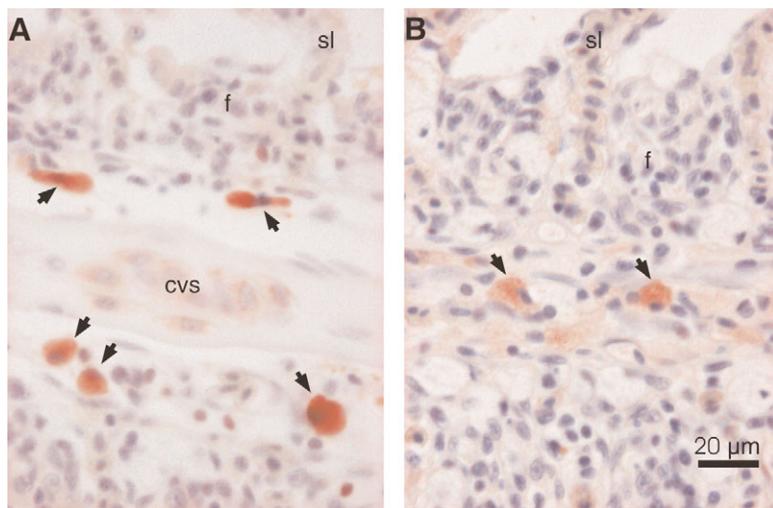


Fig. 2. Immunohistochemical demonstration of (A) CRH-positive and (B) CRH-BP-positive cells in the gills from *C. carpio*. Arrows indicate the immunoreactive cells. f, filament; sl, secondary lamellae; cvs, central venous sinus.

Table 1. Blood and plasma parameters from *Cyprinus carpio* controls and fish 3 weeks into an infection with *Trypanoplasma borreli*

| | Control | Infected with <i>T. borreli</i> |
|--|-------------------|---------------------------------|
| pH | 7.65 \pm 0.03 | 7.68 \pm 0.03 |
| [Lactate] (mmol l ⁻¹) | 4.73 \pm 0.47 | 3.43 \pm 1.26* |
| [Glucose] (mmol l ⁻¹) | 3.70 \pm 0.71 | 3.28 \pm 0.46 |
| [K ⁺] (mmol l ⁻¹) | 2.80 \pm 1.23 | 2.76 \pm 0.30 |
| [Na ⁺] (mmol l ⁻¹) | 140.78 \pm 3.65 | 136.82 \pm 4.71 |
| [Cl ⁻] (mmol l ⁻¹) | 114.78 \pm 9.60 | 112.15 \pm 4.32 |
| [Cortisol] (ng ml ⁻¹) | 3.71 \pm 1.09 | 21.67 \pm 28.73 |
| Haematocrit (%) | 39.69 \pm 2.67 | 26.74 \pm 4.02* |
| Leucocrit (%) | 0.87 \pm 0.27 | 1.62 \pm 0.31* |

Values are means \pm s.d. ($N=6$).
*Significant difference from the controls ($P<0.05$).

Regulation of CRH-BP and CRH-R1 in gills and skin after infection and stress

Infection experiment with *T. borreli*

Three weeks following infection, the number of parasites in the blood was counted and ranged between 9×10^6 and 7.5×10^7 parasites/ml blood in the infected group. There were no parasites present in the control fish. The values obtained for blood parameters (haematocrit, leucocrit and plasmatic variables) are listed in Table 1. The haematocrit was lower in the infected group than in the control group ($P<0.05$), while the leucocrit was higher in the infected group ($P<0.05$). There was no significant difference in the mean plasma cortisol levels between the control and infected group, although cortisol levels fluctuated markedly between infected, but not control individuals. The lactate concentration in the plasma was lower in the infected group ($P<0.05$) than in the control group. There was no significant difference in the concentration of Na⁺, K⁺, Cl⁻, glucose and pH between the control and infected groups.

Expression of CRH-BP and CRH-R1 mRNA in the gills was significantly lower ($P<0.05$) in the infected group compared with the non infected group (Fig. 5A). In the skin a similar pattern was observed but the effects were not statistically different (Fig. 5B).

Restraint

The values obtained for blood parameters from the 24 h restraint experiment are listed in Table 2. Haematocrit values and the concentration of cortisol and glucose were higher ($P<0.05$) in the stressed compared with the control group, while the Na⁺ concentration was lower ($P<0.05$) in the stressed group. The concentration of K⁺ and lactate did not differ between both groups.

Similar to the infection experiment, expression of CRH-BP and CRH-R1 was lower ($P<0.05$) in

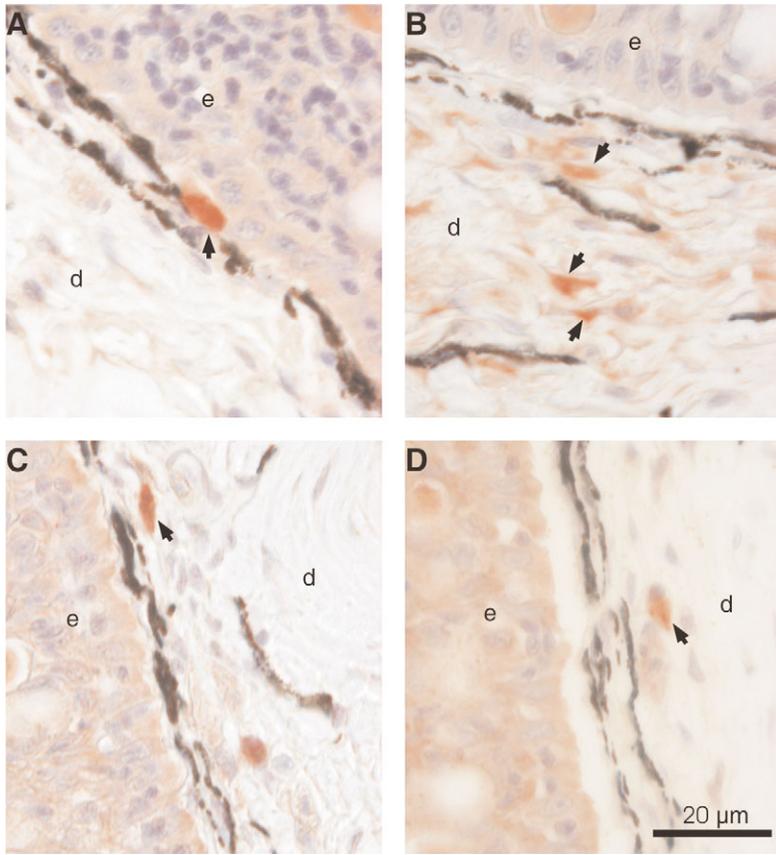


Fig. 3. Immunohistochemical demonstration of (A,B) CRH-positive and (C,D) CRH-BP-positive cells in the skin from *C. carpio*. Arrows indicate the immunoreactive cells. e, epidermis; d, dermis.

the stressed compared with the control group, in both gills and skin (Fig. 6). This reduced gene expression is paralleled by a lower number of CRH-BP-positive cells in the gills in the stressed than in the control group ($P < 0.05$; Fig. 6B). The total number of macrophage-like cells was also lower in the stressed group, although this difference was not statistically significant. The expression of ribosomal 40S and α -actin was unaffected by infection or 24 h restraint.

over 50%, independently of the functional activity of the HPI axis (Schafer et al., 1996, 1997). In contrast to these studies, which suggest a suppressive effect of CRH on local immune activation, intradermally injected CRH induces local, CRH-R1-dependent mast cell degranulation and increases vascular permeability (Theoharides et al., 1998). Collectively, these studies indicate that the role of the cutaneous CRH system is complex, and that the balance between its activation and

Discussion

We have established that the CRH-BP and CRH-R1 genes are expressed in the gills and skin of common carp. Expression of their cognate ligand CRH is detectable in both organs, albeit at a markedly lower level. By immunohistochemistry it was substantiated that gene expression of CRH and CRH-BP occurs, at least in part, in macrophage-like cells in the tissue of gills and skin. Moreover, gene expression of CRH-BP and CRH-R1 markedly drops in response to acute stress or infection, two different situations that both involve imminent or ongoing disturbance of homeostasis.

In mammals, the presence of a cutaneous CRH system is well established. This system responds to diverse stimuli such as immune cytokines, UV radiation and skin pathology, in which the common denominator appears to be local damage (Slominski et al., 2000). Yet, the local effects of CRH in mammalian skin are diverse. CRH inhibits IL-1 α -induced prostaglandin synthesis, presumably *via* inhibition of cyclo-oxygenase and phospholipase A₂ (Fleisher-Berkovich et al., 1998). Moreover, direct topic application of CRH to cutaneous or mucosal tissue evoked vasoconstrictive and anti-inflammatory effects (Wei and Thomas, 1994; McLoon and Wirtschafter, 1997; Gjerde et al., 1998). CRH injected subcutaneously or intravenously into rats with thermal injury reduced local fluid accumulation in injured skin of treated animals by

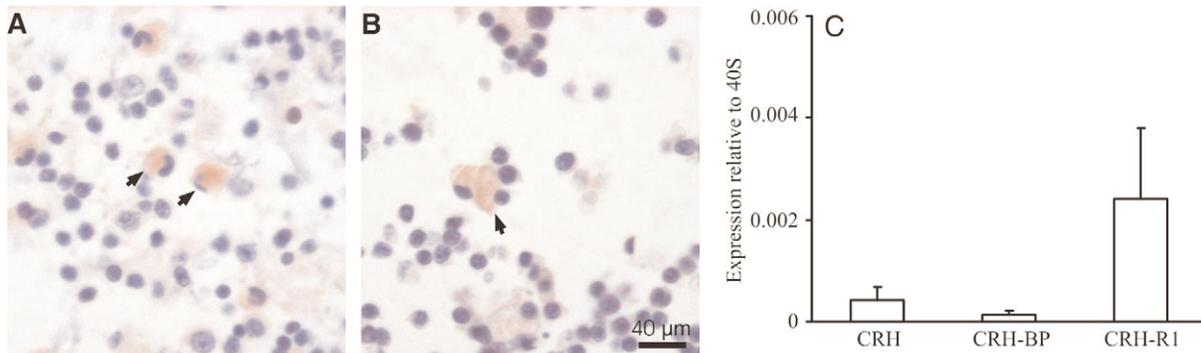


Fig. 4. (A) CRH immunoreactive cells and (B) CRH-BP immunoreactive cells in the phagocytic fraction from *C. carpio* gills. Arrows indicate the macrophage-like immunoreactive cells. (C) Expression of CRH, CRH-R1 and CRH-BP in the phagocytic fraction from carp gills. Expression was assessed by RQ-PCR and is plotted relative to the expression of ribosomal 40S. Values are means \pm s.d. ($N=4$).

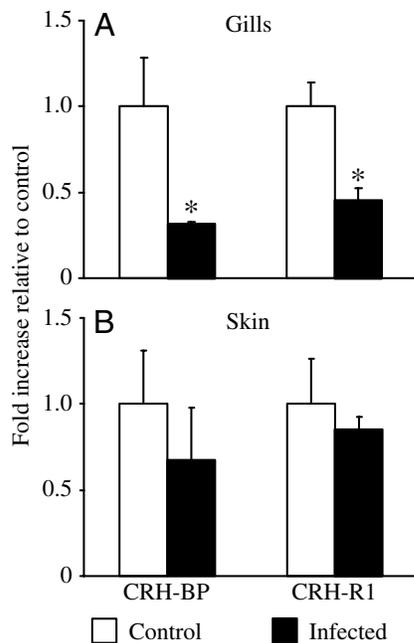


Fig. 5. CRH-R1 and CRH-BP expression levels in (A) gills and (B) skin from *C. carpio*, 3 weeks after infection with *T. borreli*. Values are means \pm s.d. ($N=5$). Asterisks indicate significant differences from the controls ($P<0.05$). Expression is standardised for ribosomal 40S expression and the relative quantification value is expressed as $2^{-\Delta\Delta Ct}$.

inhibition may depend on an interplay with many local as well as systemic parameters.

Acute restraint stress and infection with a blood parasite had marked effects on different physiological processes. These physiological changes were in line with expectations, i.e. changes in leucocrit after infection, and higher cortisol and glucose levels after stress. In addition we have observed that the tissues composing the external surfaces of carp express all the major components of a local CRH system, which is reminiscent of the mammalian skin CRH system. The general response of this integumental CRH system to stress and infection is similar. This suggests involvement of diverse local and systemic signals in the local tissue response. The downregulation of CRH-BP and CRH-R1 in gills and skin in

Table 2. Blood and plasma parameters from *Cyprinus carpio* controls and following 24 h net restraint

| | Control | Restraint |
|--|-------------------|---------------------|
| pH | 7.81 \pm 0.04 | 7.79 \pm 0.03 |
| [Lactate] (mmol l ⁻¹) | 3.96 \pm 0.81 | 3.04 \pm 0.83 |
| [Glucose] (mmol l ⁻¹) | 2.59 \pm 0.86 | 17.46 \pm 4.1* |
| [K ⁺] (mmol l ⁻¹) | 3.71 \pm 0.47 | 3.99 \pm 0.68 |
| [Na ⁺] (mmol l ⁻¹) | 137.00 \pm 1.68 | 127.00 \pm 2.48* |
| [Cortisol] (ng ml ⁻¹) | 33.49 \pm 22.22 | 219.57 \pm 94.55* |
| Haematocrit (%) | 35.05 \pm 2.89 | 39.05 \pm 1.95* |

Values are means \pm s.d. ($N=8$).

*Significant difference from the controls ($P<0.05$).

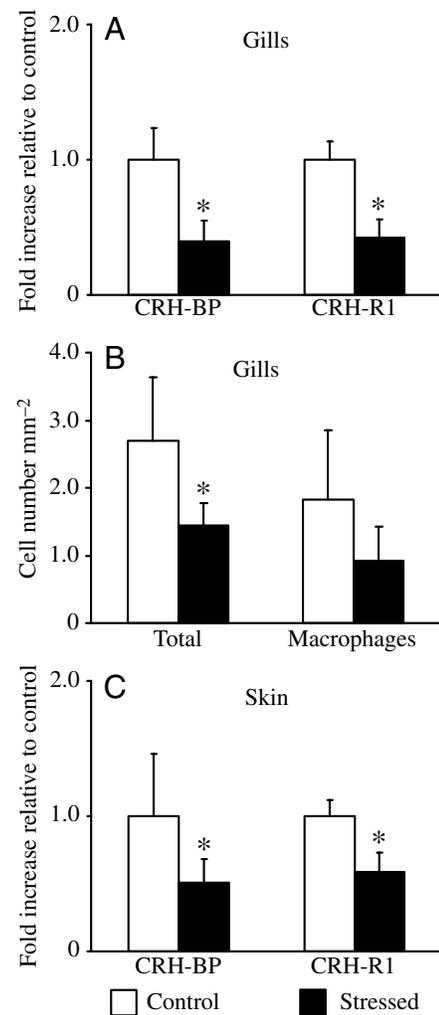


Fig. 6. (A) CRH-R1 and CRH-BP expression levels in gills, (B) the total number of CRH-BP immunopositive cells and (C) CRH-R1 and CRH-BP expression in skin of *C. carpio* following 24 h restraint. Values are means \pm s.d. ($N=5$). Asterisks indicate significant differences from the controls ($P<0.05$). Expression is standardised for ribosomal 40S expression and the relative quantification value is expressed as $2^{-\Delta\Delta Ct}$.

response to acute systemic stress suggests that the transiently elevated plasma cortisol levels exert a negative feedback on peripheral CRH-R1 and CRH-BP expression, as was reported earlier for carp pituitary CRH-R1 expression in the same stress paradigm (Huisling et al., 2004). Nonetheless, the similar inhibition of gill and skin CRH-BP and CRH-R1 expression observed in the absence of significantly elevated plasma cortisol levels during *T. borreli* infection suggests that the expression of the genes comprising the skin and gill CRH system is locally regulated. Alternatively, given that CRH and CRH-BP immunoreactivity is at least in part associated with macrophage-like cells, changes in gene expression may reflect redistribution of immune cells. Redistribution of immune cells in response to acute stress (Huisling et al., 2003) or infection with *T. borreli* (Scharsack et al., 2003) have been reported

earlier in carp and is considered to contribute significantly to the local immune surveillance.

In carp gills and skin, expression of CRH is detectable but low, which is reminiscent of the difficulties in detection of CRH gene expression in the skin of mice (Slominski et al., 1996; Slominski and Wortsman, 2000). Although there are ample CRH immunoreactive cells in both gills and skin, the markedly lower CRH expression compared to the expression of CRH-BP and CRH-R1 suggests that the local CRH system responds to systemic CRH or, alternatively, to CRH-related peptides. We previously observed pronounced immunoreactivity for CRH and CRH-BP in the pituitary pars nervosa and suggested their involvement in the regulation of the release of one or several pituitary pars intermedia peptides (Huising et al., 2004). Alternatively, systemic CRH from the pituitary pars nervosa may be released directly into circulation and act on peripheral CRH receptors in gills and skin. Another possible source of ligand is the caudal neurosecretory system or urophysis, which contains and releases considerable quantities of urotensin-I, and, in flounder, has very high expression of CRH (Lu et al., 2004). Urotensin-I is a peptide related to CRH that is capable of binding to both CRH-R1 and CRH-BP (Vaughan et al., 1995; Behan et al., 1989). Urophysial urotensin-I was initially discovered for its osmoregulatory capacity, and the fish gills would form a logical target organ for such signal.

We must also consider the possibility that the local CRH system in gills and skin can be directly and autonomously activated by an external stress. Gills and skin are strategically located facing the external and internal environments, and are permanently exposed to stresses and pathogens. These factors in combination with the key functions that are united in the fish integument require a constitutive mechanism to deal with stresses while cellular/tissue damage is still confined and of low magnitude, i.e. before the systemic stress response is triggered. The observation of CRH-positive cells in the basal layer is in line with such a mechanism, as basal layer cells have previously been reported to protect against high copper exposure from the water (Dang et al., 1999).

In summary, we have presented evidence for the existence of a local CRH system in teleost fish. This system is responsive to acute systemic stress as well as to prolonged infection with the blood flagellate *T. borreli*. Therefore, we consider this system analogous to the cutaneous CRH system that has been reported for human and rodent species. Given the presence of similar systems in evolutionary distant vertebrate phyla such as fish and mammals reinforces the importance of a local cutaneous CRH system for the proper and rapid response to various biological, chemical and physical hazards.

List of abbreviations

| | |
|--------|--|
| CRH | corticotropin-releasing hormone |
| CRH-BP | CRH-binding protein |
| CRH-R1 | CRH-receptor 1 |
| HPI | hypothalamus–pituitary–interrenal axis |

| | |
|--------|--|
| POMC | pro-opiomelanocortin |
| RIA | radioimmunoassay |
| RQ-PCR | real time quantitative polymerase chain reaction |
| RT | reverse transcriptase |
| TMS | tricaine methane sulphonate |

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