A differential role for corticosteroid receptors in neuroendocrine-immune interactions in carp (*Cyprinus carpio* L.)

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'Every communication has a content and relationship aspect such that the latter classifies the former and is therefore a metacommunication.'

Paul Watzlawick, communication theoretician

1

Introduction: neuroendocrine-immune interactions in teleostean fishes

Ellen H. Stolte

parts of this chapter will be used for an invited contribution to Bernier, N. and van der Kraak, G. (eds.). *Fish neuroendocrinology*, Elsevier

1.1 Neuroendocrine-immune interaction

A body's balanced internal milieu guarantees constancy of proper cell functions and allows for the coordinated physiological processes that determine the adaptability of the organism required to interact dynamically with the ever changing environment. Maintenance of the balanced internal milieu is in fact based on a dynamic equilibrium of bidirectional processes (*e.g.* anabolism and catabolism, influxes and effluxes, *etc.*). If the bandwidth of the equilibrium processes is narrow we refer to this maintenance process as homeostasis, if a wider bandwidth applies, a physiological process is better defined by the term allostasis, also defined as constancy through changes (McEwen and Wingfield, 2003; Wingfield, 2005). Stress is defined as the condition of an animal or organism, where potential negative, intrinsic or extrinsic, stimuli (stressors) of physical, chemical or biological (pathogenic) nature threaten the dynamic homeostatic or allostatic equilibria (Korte *et al.*, 2005; McEwen, 1998). Stress may result from a less pleasant, more psychological event at one end of the spectrum to a direct physical or (bio-)chemical impact, and life-threatening diseases at the other end. Some twenty years ago, Blalock proposed that the immune system served a sensory role, a 'sixth sense' to detect pathogenic stressors that the body otherwise could not hear, feel, smell or taste (Blalock, 1984). After detection of such a stressor, the information is signaled to the nervous system to launch an appropriate endocrine response, a requirement for the neuroendocrine system to be able to communicate with the immune system and respond to immune signals. Reciprocally, it was found that stress modifies immune responses in all vertebrates, from fish to humans (Elenkov and Chrousos (2006); Fast *et al.* (2008) and refs therein). The notion and consensus thus are that a bidirectional communication between the neuroendocrine- and immune system are at the basis of successful stress coping (Ader *et al.*, 1995; Butts and Sternberg, 2008).

To communicate disequilibrium and coordinate an appropriate stress response, a common language for the neuroendocrine and immune system is required that is based on biochemical signaling understood by the cells of both these systems. This communication depends on direct and fixed neural pathways, and humoral (endocrine, paracrine, autocrine) messages; the latter messengers concern mainly proteins, peptides and steroids. For these messengers a plethora of receptor mechanisms and intracellular pathways is utilised by target cells to elicit distinct and specific responses. Signals of endocrine glands and cells are called hormones; messengers secreted into the circulation to inform distant targets. Immune tissues and cells produce signals that are called cytokines, signals that for instance affect leukocyte proliferation or differentiation and may exert actions on shorter distances

or even closely associated cell populations. Cytokines are polypeptides or glycoproteins (occasionally multiheteromers) that are generally expressed constitutively at a very low level. Typically their production is transient and actions are usually local (auto-, para- and intracrine rather than endocrine). Hormones are often produced and stored in significant amounts in an endocrine cell or tissue and directed to restricted, receptor-defined target cells to specify the elicited responses. Cytokines are considered to be produced by many different cell types, and to be pleiotropic in their action, *i.e.* they often elicit multiple effects in different target cells and may show functional redundancy (Vilçek, 2003). However, the classical and (too) strict concept that puts hormones *versus* cytokines requires amendments: many signaling molecules classically referred to as either hormone or cytokine may belong (as gene analyses unequivocally establish) to the same molecular family of structurally related proteins. Members of these families have evolved from common ancestors through series of successive duplication events. The level of sequence conservation varies greatly and sequence identity for that reason is of limited value in establishing relatedness (Ottaviani *et al.*, 2007; Huising *et al.*, 2006a; Bird *et al.*, 2006).

The endocrine and the immune systems must coordinate their activities to ensure proper and optimal stress coping, wound healing and coping with pathogenic invasion (*e.g.* a pathogenic challenge is a crucial extrinsic stressor, which has to be communicated to the rest of the body). Both systems have to be kept in balance; an inflammatory response is necessary to overcome a bacterial infection, but may cut as a doubly edged sword, as too strong inflammatory responses can lead to destruction of host tissues. Importantly, stress-induced energy redistribution as a result of enhanced cortisol activity may result in suppression of immune functions that are of second order importance when survival is at stake (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997).

The worldwide growing demand for fish to provide the human population with protein has resulted in intensive aquaculture practices that are rapidly expanding over the last few decades. Unfavorable conditions that come with intensive fish farming may include high rearing density and unnatural housing and social situations. Moreover, frequent handling, transport, overfeeding and suboptimal water quality (*e.g.* variable temperatures, high concentrations of waste products) are conditions that individually or combined will evoke stress responses in the fish that will interfere with proper immune responses (Terova *et al.*, 2005; Yada and Nakanishi, 2002; Fast *et al.*, 2008). The conditions associated with intensification of fish-farming practices such as high rearing densities and the movement of broodstock facilitate rapid pathogen expansion that increases the risk of infectious diseases. Prevention and/or effective treatment of infectious diseases requires understanding of the presumed

elaborate bidirectional communication with shared ligands and receptors that must be at the basis of proper neuroendocrine and immune functioning.

1.2 Immunity in fishes

Fish are, in particular via their gills, in intimate contact with their aqueous environment that harbors high numbers of pathogens. Despite this potentially high pathogen exposure the extant teleostean fishes are among the evolutionary oldest vertebrates (the first bony fishes appeared around 450 million years ago) and are equipped with a well-developed and effective immune system. First, they produce a biochemical barrier to prevent pathogen invasion, viz. the integumental mucus layer, which contains many bactericidal peptides, lysozyme, lectins and proteases (reviewed by Magnadottir (2006) and Ellis (2001)). Second, when pathogens pass this barrier, an array of soluble and cellular defense mechanisms is at hand. Fish are the earliest vertebrates that have developed both arms of the immune system, reflected by the innate and the adaptive immune response (figure 1.1). The innate immune system functions with 'generic' recognition of pathogens and prevention of pathogen dispersal. The adaptive immune system specifically recognises pathogens on the basis of specific surface antigens and clears an infection via production of specific antibodies and cytotoxic lymphocytes; importantly, this system will generate a memory. Third, the combined immune responses are regulated to be rapidly terminated after pathogen clearance to prevent damage to the host.

1.2.1 Immune organs and cytokines in fishes

Fish lack bone marrow as a primary immune organ, but their haematopoietic cells reside in the so-called head kidney (embryologically a pronephric structure, located in the frontal section of the peritoneum, often closely behind the gills) and gives rise to both myeloid and lymphoid immune cells (van Muiswinkel, 1995). Fish lack lymph nodes as secondary immune organs. The spleen and the head kidney are sites for interaction of the immune system with antigens and harbor the antibody producing lymphocytes. The thymus, as in mammals, acts in fish as a centre of T-lymphocyte maturation. The immune system associated with epithelial surfaces of the gut, gills and skin form the first line of defense and the connective tissues associated are densely populated with immune cells to attack any penetrating infectious agent; to this end the immune cells often penetrate the epithelial layers. As mentioned above, immune cells communicate via cytokines and associated receptors, which is critically important for a fast and effective attack on pathogens. Only minute

amounts of cytokines are required to generate strong pro- and anti-inflammatory effects. A tight control over this cytokine production guarantees prevention of cell damage. Dependent on the stage of infection and function of particular cell types, immune cells produce different types from an array of cytokines (at least a dozen different cytokines have been described in fish and many more are predicted to be found). Among these cytokines we distinguish interleukins (ILs) such as IL-1β (Zou *et al.*, 1999), IL-4 (Li *et al.*, 2006a), IL-6 (Bird *et al.*, 2005b), IL-10 (Zou *et al.*, 2003; Savan *et al.*, 2003) and IL-12 (Huising *et al.*, 2006c), that serve communication between leukocytes. Also tumor necrosis factor alpha (TNF-α) (Hirono *et al.*, 2000) and interferon gamma (IFN-γ) (Zou *et al.*, 2004) belong to the family of cytokines. Moreover, chemokines, an acronym for chemo-attractant cytokines coordinate cell chemotactic migration (Lee *et al.*, 2001; Huising *et al.*, 2003b). Cytokines elicit a very broad range of actions on cell growth and differentiation and for that reason are often subdivided into growth factors, pro-inflammatory and anti-inflammatory cytokines. The differential kinetics of cytokine expression and secretion in time after detection of the pathogen ensures effective clearing of the pathogen whilst minimising the damage to the host.

1.2.2 Innate immunity: cell types

The principal cell types of the innate immune system of teleostean fishes are the myeloid cells, neutrophilic granulocytes and macrophages (from monocyte precursor), cells that all have phagocytosis as major function. These cell types migrate to a site of infection / inflammation / pathogen invasion and kill bacteria and infected cells with microbicidal nitrogen and oxygen radicals (respiratory burst; reviewed by Neumann *et al.* (2001)). Killing of infected cells is performed by non-specific or natural cytotoxic cells (NCC), the fish equivalent of the mammalian natural killer (NK) cells (Shen *et al.*, 2002; Fischer *et al.*, 2006), or by production of bactericidal or lytic proteins/enzymes (Magnadottir, 2006).

1.2.3 Innate immunity: pathogen recognition

Pathogens have particular characteristics important for virulence, which are prone to mutation and are usually not expressed in the host. These rather invariable characteristics are collectively named pathogen associated molecular patterns (PAMPs), such as fungal β1,3-glucan, viral double stranded RNA or bacterial cell wall products such as lipopolysaccharide (LPS). They are recognised by pathogen recognition receptors (PRRs). PRRs exist as soluble, humoral variants, like the complement protein C3 or are expressed as membrane

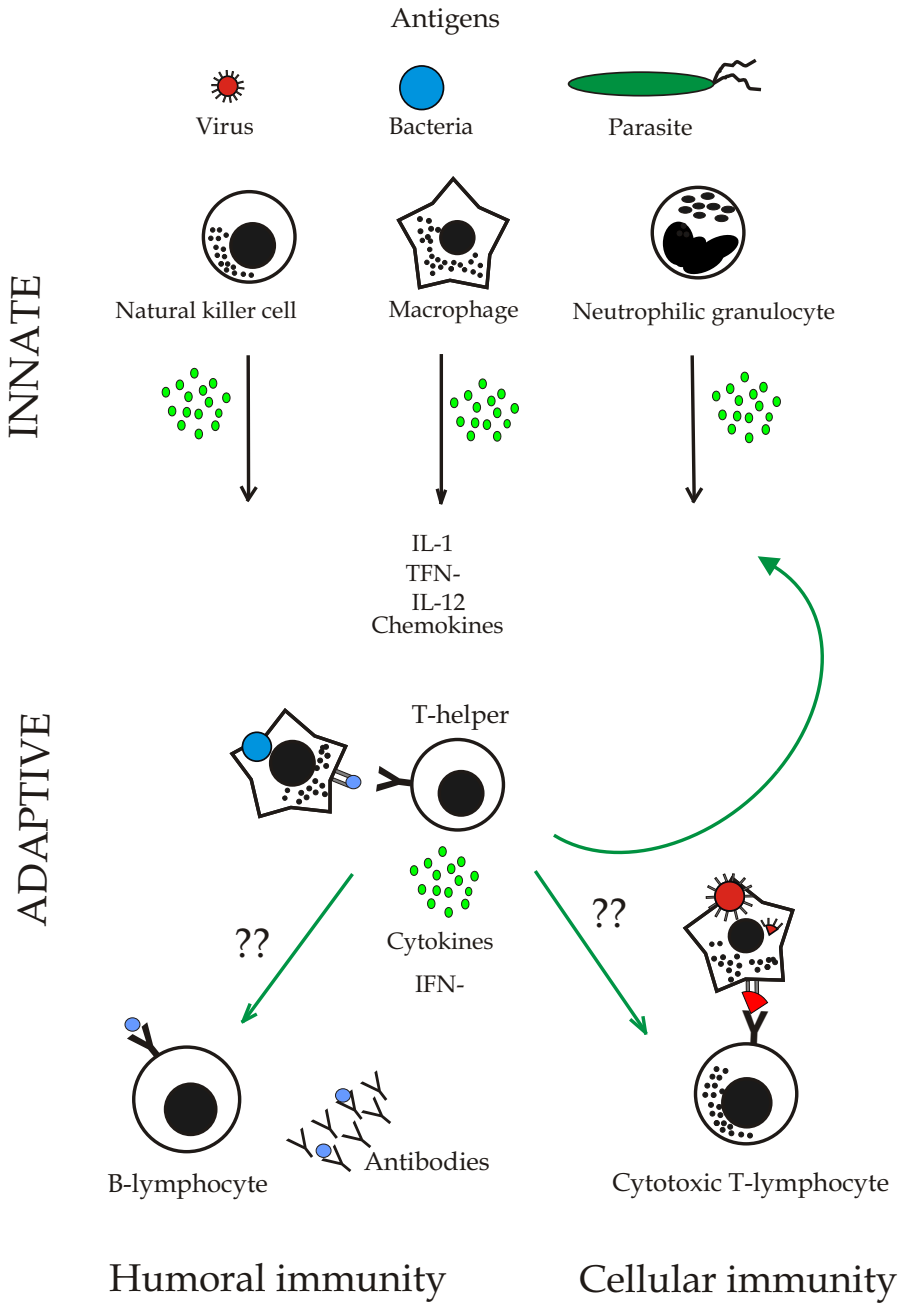

Figure 1.1: Schematic representation of the immune system. See text for details. Briefly, by neutrophils, macrophages and natural cytotoxic cells will directly attack and kill these antigens and produce pro-inflammatory cytokines. After killing, antigen presenting cells will display part of the pathogen on their cell surface. This is recognised by B-lymphocytes and will lead to antibody production. If a T-lymphocyte recognises such a displayed pathogen it leads to clonal expansion of these T-lymphocytes, which will differentiate into cytotoxic T-lymphocytes that will kill pathogen infected cells. It has not yet been established if teleostean T-lymphocytes are also able to differentiate into T-helper cells that support B-lymphocyte function. Abbreviations: IL-1β; interleukin-1 beta, TNF-α; tumor necrosis factor alpha, IL-12; interleukin-12, IFN-γ; interferon gamma.

receptors in cells of the immune system, such as many of the Toll-like receptors (TLRs; Medzhitov *et al.* (1997)). In mammals, particular TLRs recognise a distinct group of infectious agents (*e.g.* TLR1 and TLR2 principally recognise lipoprotein and peptidoglycans from gram-positive bacteria, TLR3 recognises double-stranded RNA from viruses, TLR4 recognises lipopolysaccharide (LPS) of gram-negative bacteria *etc.*; reviewed by Krishnan *et al.* (2007)). Analysis of the zebrafish genome reveals orthologues for most TLRs expressed in mammals (Meijer *et al.*, 2004; Roach *et al.*, 2005). Moreover, stimulation of fish leukocytes with TLR specific ligands resulted in cytokine responses which corroborate the biological function of these receptors (Purcell *et al.*, 2006).

When activated, these pathogen recognition receptors induce a number of responses directed towards killing of the pathogen and infected cells, prevention of pathogen dispersal, and communication of type and severity of infection via release of cytokines and pro-inflammatory molecules profile. Killing of pathogens is increased by opsonisation (*i.e.* a process by which a pathogen is marked for ingestion and destruction by phagoycytic cells) and subsequent phagocytosis of the pathogen (Holland and Lambris, 2002). Moreover, microbicidal reactive oxygen species are produced by phagocytic cells (Novoa *et al.*, 1996; Verburg-van Kemenade *et al.*, 1996). Additionally, natural cytotoxic cells are stimulated to assist in killing of infected cells and the complement system is activated to attack pathogen membranes (Evans *et al.*, 2005; Magnadottir, 2006). Moreover, the acute phase response is activated, which involves a number of plasma proteins (*e.g.* serum amyloid A, transferrins, lysozyme, and α-2-macroglobulin) and limits dispersal of pathogens but also initiates tissue repair (Magnadottir, 2006). TLR activation will induce pro-inflammatory cytokine expression via intracellular pathways that are presumed to be analogous to those described in mammalian systems (reviewed by Brikos and O'Neill (2008)). Intriguingly, the TLR specificity determines the resulting cytokine profile and by doing so communicate the nature of the infection (Purcell *et al.*, 2006). For instance, a viral attack will induce the production of type I interferon-α and -β that will 'warn' other cells to increase their antiviral defenses and stimulate cytotoxic cells activity to lyse infected cells and prevent further multiplication of the virus (Robertsen, 2006).

1.2.4 Innate immune response: cytokine signaling

In the vertebrate innate immune system, pro-inflammatory cytokines TNF-α, IL-1β and IL-6 induce the acute phase response and chemokine release (Burger and Dayer, 2002). Interleukin 12 (IL-12) induces release of type II interferon: interferon gamma (IFN-γ). Here we focus on those cytokines that were studied in this thesis. TNF-α is constitutively

expressed in kidney and gill and can be induced by LPS stimulation of head kidney phagocytes (Laing *et al.*, 2001). It mediates resistance to infections by controlling intracellular pathogen replication and induces cell proliferation, leukocyte migration and increase phagocytic activity, and is also involved in production of microbicidal nitric oxide (NO) (Zou *et al.*, 2003; Ordas *et al.*, 2007; Grayfer *et al.*, 2008), presumably by regulation of inducible nitric oxide synthase (*i*NOS) expression (Saeij *et al.*, 2003b). IL-1β secreted by macrophages stimulates head kidney leukocyte proliferation, initiates an acute phase response and activates macrophages and T-lymphocytes (Hong *et al.*, 2001; Buonocore *et al.*, 2005; Lu *et al.*, 2008). IL-12 expression can be induced in head kidney macrophages after bacterial and virus infections and is suggested to increase the cytolytic properties of natural cytotoxic cells and T-cells to ensure clearing of virus-infected cells (Huising *et al.*, 2006c; Forlenza *et al.*, 2008a; Nascimento *et al.*, 2007). IFN-γ is expressed by natural cytotoxic cells and T-lymphocytes. It activates macrophages to increase microbicidal activity and stimulates antigen presentation (Zou *et al.*, 2005; Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006). Chemokines are expressed by all classical immune organs such as head kidney, spleen, thymus, and kidney and direct leukocytes to the site of inflammation (Huising *et al.*, 2003b; Laing and Secombes, 2004).

1.2.5 Adaptive immune response: cell types, recognition, and memory

The adaptive immune response provides the vertebrate immune system with the ability to recognise and remember specific pathogens and to mount a stronger and faster response in a subsequent encounter with the same pathogen. To mount such an immune response, a specific pathogen or antigen needs to be recognised. The major histocompatibility complex (MHC) proteins act as 'signposts' and display fragmented pieces of an antigen on the cell surface of antigen presenting cells. The T-cell receptor (TCR) is a molecule found on the surface of T-lymphocytes (or T-cells) that is, in general, responsible for recognition of antigens bound to major histocompatibility complex (MHC) molecules. Recognition of a particular antigen presented on the MHC with a specific TCR will lead to clonal expansion of T-lymphocytes bearing this TCR (Rudolph *et al.*, 2006). B-lymphocytes recognise epitopes of the antigen with their specific B-cell receptor (membrane-bound immunoglobulin) and secrete specific antibodies (humoral immunity). These antibodies will attach to the pathogen, which results in increased opsonisation and phagocytosis of pathogens (Bishop *et al.*, 2003).

Antigen presentation is mediated by macrophages (Martin *et al.*, 2007) and B-lymphocytes produce antibodies and are characterised by expression of immunoglobulin at their cell

surface (Magnadottir *et al.*, 2005). Mammals display a division of T-lymphocyte functions, executed by two different types of T-lymphocytes, helper T-lymphocytes (Th) that support B-lymphocyte function and cytotoxic T-lymphocytes (Tc) that will attack and kill virus-infected cells. Th-cells are characterised by co-receptor CD4 and MHC class II (MHCII), whereas Tc-cells are characterised by CD8 and MHCI. Such a division of functions is not fully established in fish, although fish leukocytes do display cytotoxic activity (reviewed by Fischer *et al.* (2006)). However, teleostean T-lymphocytes display a TCR (Partula *et al.*, 1995; Rast *et al.*, 1995; Scapigliati *et al.*, 2000), and show expression of CD4 (Suetake *et al.*, 2004) and CD8 (Hansen and Strassburger, 2000) as well as MHC class I and II (Hashimoto *et al.*, 1990; Stet *et al.*, 2003). Interactions between MHC and TCR are currently under investigation (Randelli *et al.*, 2008; Buonocore *et al.*, 2008), and teleostean fishes display a very diverse immune repertoire (Boudinot *et al.*, 2008).

In teleostean fishes, as in mammals, a first antigen encounter will induce some long-lived memory cells that retain the capacity to be stimulated by the antigen, which results in a faster and more vigorous secondary response (Rijkers *et al.*, 1980b; dos Santos *et al.*, 2001; Kaattari *et al.*, 2002). It should be kept in mind that teleostean fishes have different immunoglobulin isotypes compared to mammals. The main immunoglobulin studied is tetrameric (in contrast to the mammalian pentameric) immunoglobulin M (IgM; (Håvarstein *et al.*, 1988; Kaattari *et al.*, 1998)). Additionally an IgD (Wilson *et al.*, 1997) and Ig heavy chain variations such as (IgZ) (zebrafish, *Danio rerio* immunoglobulin; (Danilova *et al.*, 2005)), which is called IgT (tau; for teleostean) in rainbow trout (*Oncorhynchus mykiss*)(Hansen *et al.*, 2005), and a chimera (IgM–IgZ; Savan *et al.* (2005)) have been described, but functional implications have not yet been established. Genomic analyses, however, have shown ample possibilities for recombination and rearrangements of the variable region of the immunoglobulin molecule which would enable an extended antibody repertoire (Bengtén *et al.*, 2006; Solem and Stenvik, 2006).

Antigen specific T-lymphocytes will either kill infected host cells (Fischer *et al.*, 2006) or assist antibody production of the antigen specific B-lymphocytes (by release of cytokines such as IL-1β and IFN-γ) (Cain *et al.*, 2002). This cellular immunity is also more efficient upon a second stimulation as was shown by faster rejection of second compared to first grafts (Grether *et al.*, 2004).

Although this adaptive immune response is rapid and strong upon a second encounter with the same pathogen, it takes days to weeks, in ectotherms such as fishes, depending on the ambient temperature, to be fully effective after a first encounter (Rijkers *et al.*, 1980a; van Muiswinkel, 1995). Therefore the innate immune system is of crucial importance.

Without a proper initial killing and prevention of dispersion of the pathogen, the host may succumb to the infection, long before an adaptive immune response has been mounted.

1.2.6 Termination of the immune response

After the pathogen has been dealt with, the inflammatory response needs a rapid end: production of microbicidal proteins, reactive oxygen species and circulation of high numbers of cytotoxic cells are potentially harmful also to the host and may even become fatal. Therefore, anti-inflammatory cytokines IL-10 and tumor growth factor-β (TGF-β) are produced to inhibit activation of the immune response and initiate processes of wound healing, tissue remodelling, and recovery. The peak of IL-10 expression occurs during the late phase of a inflammatory response (Pinto *et al.*, 2007) and correlates with inhibited expression of the pro-inflammatory cytokine IL-1β and chemokines (Chadzinska *et al.*, 2008; Seppola *et al.*, 2008). TGF-β is also increased at the late stage of infection (Tafalla *et al.*, 2005). Moreover, it downregulates the nitric oxide response of TNF-alpha-activated macrophages (Haddad *et al.*, 2008). Additionally, apoptosis is tightly regulated during an immune response. Apoptosis is required to maintain balance in dynamic cell populations and can initially be inhibited to increase the life span and effectiveness of phagocytic cells (Weyts *et al.*, 1998b). However, large populations of phagocytic or cytotoxic cells can be harmful and need to be swiftly cleared after termination of their initial task. Successive secretion of pro- and anti-inflammatory cytokines and balanced apoptosis result in an effective activation of the immune response and an appropriate de-activation (Elenkov and Chrousos, 2006).

1.3 The neuroendocrine system

The neuroendocrine system consists of endocrine cells in the brain that project to the pituitary gland or release hormones into the circulation (with the pituitary pars intermedia as important neurohaemal organ) (Flik *et al.*, 2006). Hormones from the brain and pituitary gland in turn induce hormone release from peripheral endocrine glands. The pituitary gland is a first and key peripheral target. In the stress axis the head kidney is the site of production of the stress hormone/steroid cortisol and catecholamines noradrenalin and adrenalin. In addition to a humoral control of peripheral endocrine cells, direct innervation of endocrine glands occurs via the autonomic nervous system. Here we will only address those elements of the neuroendocrine system that are principally involved in stress and will be discussed in this thesis.

1.3.1 Autonomic nervous system

The autonomic nervous system (ANS) connects to endocrine glands, but also to smooth muscles and to the excretory and digestive systems and to the heart. The ANS is divided in a parasympathetic and sympathetic section. The parasympathetic branch functions with actions that do not require immediate reaction, 'rest or digest'. The sympathetic nervous system plays an important role under conditions of stress and prepares the body for immediate 'fight or flight'. The teleostean head kidney receives direct sympathetic innervation. Following a stressful stimulus, the catecholamines noradrenalin and adrenalin are released by the chromaffin cells (Reid *et al.*, 1998). These are fast acting hormones that prepare the organism for the first steps of the fight/flight response.

1.3.2 Hypothalamo-pituitary-interrenal (HPI) axis

Cortisol is the end-product of the hypothalamo-pituitary-interrenal (HPI-) or stress axis the analogue of the mammalian hypothalamo-pituitary-adrenal (HPA) axis (figure 1.2. When stress signals are perceived, the hypothalamic nucleus pre-opticus produces and secretes, among others, corticotropin-releasing hormone (CRH) in a paracrine fashion near the adrenocorticotropic hormone (ACTH) cells of the pars distalis and the melanocyte stimulating hormone (MSH) cells of the pars intermedia. Also, CRH may be released into the circulation in a neurohaemal arrangement in the pars intermedia, but direct evidence for that is so far lacking (Flik *et al.*, 2006). Targets for peripheral CRH of nucleus preopticus origin remain to be established. The CRH signal is received by the CRH receptor (CRH-R1) in the pituitary pars distalis ACTH-cells (Huising *et al.*, 2004a). Subsequently, adrenocorticotropic hormone (ACTH) from the precursor molecule pro-opiomelanocortin (POMC), is released into the circulation. CRH also induces release of alpha-melanocyte stimulating hormone (α-MSH) and β-endorphins from the POMC precursor in the melanotrope cells of the pars intermedia of the pituitary gland (van den Burg *et al.*, 2005). ATCH finally, induces cortisol production and release by the interrenal cells of the head kidney into circulation.

1.3.3 Cortisol

In the circulation the bulk (80 %) of cortisol is bound to serum steroid binding globulins, which leaves only 20 % of the cortisol as unbound and bioactive (Flik and Perry, 1989). Basal plasma cortisol levels vary among different teleostean species, but usually range from 5–50 ng/ml. Plasma cortisol levels can rapidly and dramatically (over 25-fold) increase

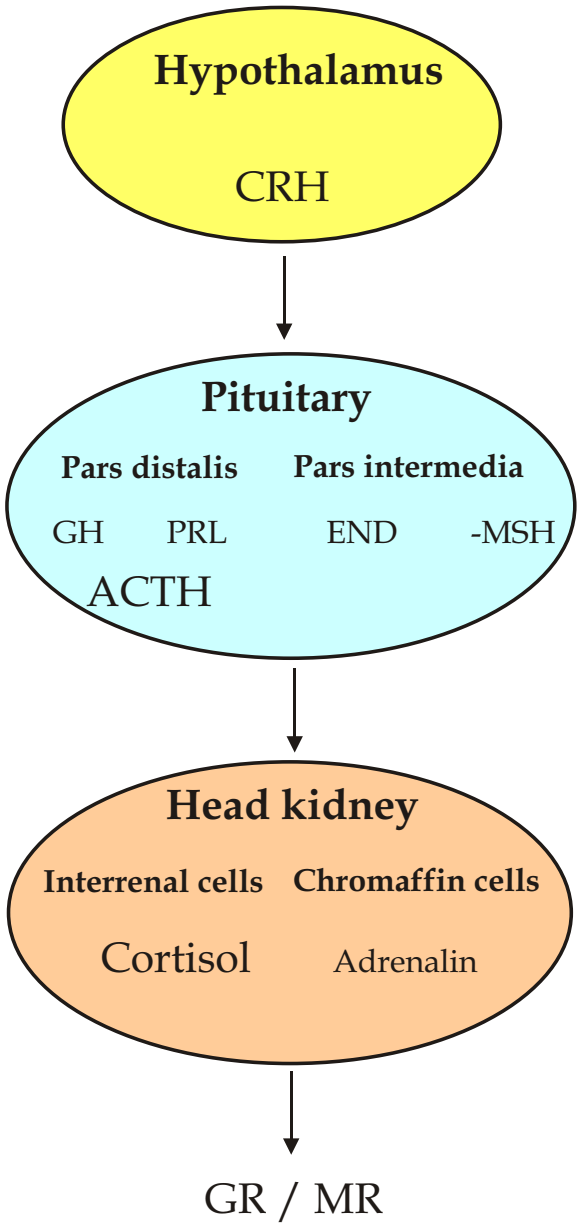

Figure 1.2: Schematic representation of the hypothalamo-pituitary-interrenal (HPI)-axis in teleostean fishes. See text for details. Briefly, CRH released from the hypothalamus induces ACTH release from the pituitary, which in its turn induces cortisol release from the interrenal cells of the pituitary. Abbreviations: CRH; corticotropin-releasing hormone, ACTH; adrenocorticotropic hormone, PRL; prolactin, GH; growth hormone, END; endorphin, α-MSH; alpha melanocyte stimulating hormone, GR; glucocorticoid receptor, MR; mineralocorticoid receptor.

and reach peak levels within 30 minutes (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997; Tanck *et al.*, 2000). However, after cessation of the stressor, plasma cortisol quickly (2 to 3 hr) drops again to basal levels. Moreover, once released into circulation, cortisol is converted and inactivated to cortisone, which lacks (the immune) regulatory properties of cortisol (Weyts *et al.*, 1997). As a cholesterol-derivative, cortisol is strongly hydrophobic and is assumed to diffuse across cell-membranes (or to be transported by as yet undefined carriers).

In the cell, cortisol is bound by one of the various intracellular receptors, for instance a glucocorticoid receptor (GR). For this binding to occur, heat shock proteins Hsp70 and Hsp90 are both essential as chaperones to open the steroid-binding cleft (Pratt and Toft, 1997). This GR-cortisol complex then translocates into the nucleus where it binds to one of a plethora of glucocorticoid responsive elements (GREs) on the DNA, to activate or repress transcription of effector genes (Stolte *et al.*, 2006). The specificity of this process is only very poorly understood.

1.4 Bidirectional communication: shared ligands and receptors

As mentioned above, maintenance of the balanced internal milieu is in fact based on a dynamic equilibrium of bidirectional processes (Yada and Nakanishi, 2002). In an immune response a delicate balance between pro-inflammatory and anti-inflammatory effects is required to effectively kill the pathogen, whilst minimising the damage to the host. Endocrine signaling, and especially the immune suppressive effects of cortisol and immune stimulating effects of growth hormone and prolactin are suggested to be involved in creating this balance (Borghetti *et al.*, 2006; Dimitrov *et al.*, 2004; Yada *et al.*, 2004). Reciprocally, cytokines released by immune cells, signal neuroendocrine, autonomic, limbic and cortical areas of the CNS to affect neural activity and modify behavior (including sleep), hormone release and autonomic function (Lorton *et al.*, 2006).

1.4.1 Hormones and hormone receptors in the immune system

Hormones that were originally believed to be of mere brain or pituitary origin have now been shown to be produced by cells of the immune system as well (Elenkov and Chrousos (2006) and refs therein). Moreover, cells of the immune system are equipped with hormone receptors, which enable endocrine, paracrine, autocrine, and intracrine regulation.

In teleostean fishes, CRH immunoreactivity was found in macrophage-like cells of the gill and skin (de Mazon *et al.*, 2006) and ACTH was demonstrated in the thymus of goldfish, *Carassius c. auratus* (Ottaviani *et al.*, 1995). Growth hormone (GH) expression has been confirmed in fish leukocytes (Yada, 2007). A variety of classical hormone receptors have been demonstrated in the fish immune system, including receptors for the HPI-axis hormones, growth hormone (GH) and prolactin (PRL). Presence of hormone receptors is all too often established so far only by effects observed after hormone treatment. Alpha-MSH stimulates phagocytosis and induces production of superoxide anions in macrophages (Watanuki *et al.*, 2003). Pituitary growth hormone (GH) and prolactin enhance radical oxygen production and induce proliferation of head kidney leucocytes (Yada *et al.*, 2004). Administration of GH enhances cytotoxic and phagocytic activities and activates immunoglobulin production (Yada, 2007). And catecholamines noradrenalin and adrenalin decreased phagocytic activity in splenic macrophages through beta-adrenergic receptor, whereas superoxide production was stimulated via the alpha-adrenergic receptor (Roy and Rai, 2008).

Glucocorticoid receptors have been described in immune cells based on messenger RNA expression (Vizzini *et al.*, 2007), steroid binding studies (Maule and Schreck, 1990; Weyts *et al.*, 1998c) and immunohistochemistry (Vazzana *et al.*, 2008). The effects of cortisol on immune cell function can be immune suppressive as well as stimulatory, depending on the type, dose response effects and duration of the stressor experienced. It was demonstrated that cortisol inhibits LPS-induced expression of acute phase proteins and pro-inflammatory cytokines and molecules (Huising *et al.*, 2005; Saeij *et al.*, 2003c). Moreover, respiratory burst activity and phagocytosis of head kidney leucocytes was significantly depressed after *in vitro* cortisol-treatment (Esteban *et al.*, 2004; Law *et al.*, 2001; Vizzini *et al.*, 2007). Additionally, cortisol was shown to inhibit proliferation and induce apoptosis of lymphocytes *in vitro* and these responses were GR-dependent, as addition of the GR-blocker RU486 reversed the effects (Pagniello *et al.*, 2002; Saha *et al.*, 2003; Verburg-van Kemenade *et al.*, 1999; Weyts *et al.*, 1997). In contrast, cortisol-treatment inhibited (GR-dependent) apoptosis in neutrophilic granulocytes of common carp, *Cyprinus carpio* (Weyts *et al.*, 1998b). *In vivo* results corroborated these *in vitro* experiments; stress reduced the number of circulating B-lymphocytes and decreased antibody responses after immunisation (Verburg-van Kemenade *et al.*, 1999), whereas the relative percentage of circulating granulocytes nearly doubled (Engelsma *et al.*, 2003a; Huising *et al.*, 2003a). As neutrophils belong to the first line of defence, it can be beneficial in situations of acute stress (and possible injury) to prolong their lifespan and maintain a higher number of circulating neutrophils.

A delicate balance between immune stimulation and suppression is hypothesised from

this widespread presence of hormones and receptors on immune cells. *In vitro*, PRL and GH attenuated the decrease in proliferation due to cortisol-treatment, and PRL inhibited cortisol-induced apoptosis (Yada *et al.*, 2004). These results suggest that the immunostimulatory properties of PRL and GH are involved in the maintenance of specific immune functions in fish through a prevention of immune suppression by cortisol.

1.4.2 Cytokines and cytokine receptors in the neuroendocrine system

The effects of cytokines on neuroendocrine functions were not extensively studied, but it has been shown that peripheral cytokines can reach the brain. Moreover cytokine receptors in the brain were reported, based on *in vitro* effects after cytokine treatment. Cytokines that affect neuroendocrine function are mostly produced by immune-related cells in the brain, viz. astrocytes or glia cells (Churchill *et al.*, 2006; Garden and Moller, 2006; Kelley *et al.*, 2003). To exert a central action, signals produced by peripheral immune organs, must first pass the blood-brain-barrier, which is leaky, particularly in the circumventricular organs. Indeed, in mammals pro-inflammatory cytokines IL-6, IL-1β, IL-12, TNF-α and IFN-γ (Di Cornite *et al.*, 2007; Muller and Schwarz, 2007) are able to reach central hormone producing cells and affect their secretion (Brunton and Russell, 2008). IL-1β and to a lesser extent TNF-α were found to induce ACTH secretion (Dunn, 2006) and intraperitoneal injection of IL-1β induced noradrenalin release from the paraventricular nucleus (Mohankumar and Quadri, 1993). In mice, intraperitoneal injection of IL-6 induced increased plasma levels of ACTH and corticosterone (Wang and Dunn, 1998). These cytokine effects on neurotransmitter and hormone secretion appear not unique for mammals as in fish IL-1β increased β-endorphin and α-MSH release from the pituitary *in vitro* (Metz *et al.*, 2006) in common carp, and elevated cortisol levels in rainbow trout *in vivo* (Holland *et al.*, 2002). Although this confirms the presence of IL-1β receptors, *in vivo* experiments are warranted to confirm this notion. Together these results suggest that cytokines can indeed affect central as well as peripheral hormone secretion. They communicate a disequilibrium (*e.g.* due to pathogen invasion) and thereby help coordinate an appropriate stress and immune response.

1.5 Thesis outline

Teleostean fishes are the oldest extant vertebrates and therefore provide from an evolutionary point of view an interesting model for comparative studies. They possess well-developed immune and neuroendocrine systems and require extensive communication bet-

ween these systems for allostasis. In this thesis we investigated the receptors for the steroid hormone cortisol, the glucocorticoid receptors (GRs) and the mineralocorticoid receptor (MR), and their involvement in stress and immune regulation. Moreover, we set out to find the pro-inflammatory cytokine IFN-γ to enable determination of its effect on (stress) hormone secretion. Furthermore, we used a genome wide screen to find additional genes that might be involved in regulation of the stress or the immune response.

1.5.1 Function of glucocorticoid receptors in stress and immunity

After a review of the available literature in **chapter 1** and thorough investigation of genome databases of several teleostean fish species, we describe the evolution of glucocorticoid receptors in **chapter 2**. We show that, as a result of a fish lineage specific genome duplication, teleostean fishes have duplicated GR genes. Both resulting proteins (GR1 and GR2) are expressed and functional in extant teleostean fishes. Moreover, we found that the mineralocorticoid receptor (MR) can also bind cortisol. Together, fish have three receptors for a single ligand, which gives ample possibilities for differential regulation in stress and immunity. In **chapter 3**, we therefore investigated the specific functional characteristics of GR1, GR2 and MR, and measured their respective senstitivity for cortisol. We found that GR1 is a relatively 'insensitive' or 'stress' receptor, which can only be activated once stress levels of cortisol have been reached, whereas GR2 is a 'sensitive' receptor that is already activated at basal levels of cortisol as occur in non-stressed fish. We subsequently established their gene expression levels in the stress axis organs (brain, hypothalamus and pituitary. Similarly, we determined receptor expression in the phagocytes and lymphocytes in **chapter 4**. Moreover, we determined a specific role of these receptors, by investigation of the effects of basal and stress levels of cortisol on expression of pro-inflammatory cytokines. Besides different ligand sensitivity, regulated receptor expression after infection may reflect specific receptor functions. Therefore we determined receptor mRNA levels after an *in vitro* cortisol-treatment or after *in vivo* infection in **chapter 5**. Concomitantly, we investigated regulation of heat shock proteins during stress or infection as these play a role in formation of the ligand-receptor complex.

1.5.2 Immune signaling to the neuroendocrine system

Unprejudiced genome wide screen approaches are required to find genes involved in bidirectional neuroendocrine-immune communication. In **chapter 6** we describe a preliminary microarray experiment in head kidney in which we investigated which of the 9000 randomly

picked cDNA clones were differentially regulated by either a parasite infection or 24 hr restraint stress.

The key pro-inflammatory cytokine IFN-γ is hypothesised to affect neurotransmitter and hormone release and plays an important role in immune responses, but had not been investigated in carp. In **chapter 7** we characterised the common carp (duplicated) IFN-γ genes and investigated expression profiles in different immune cells after *in vitro* stimulation or *in vivo* infection. We compared structural and functional characteristics to other vertebrate IFN-γ genes to hypothesise about a possible differential role for these cytokines in B- and T-lymphocyte function respectively. Finally, in **chapter 8** a synthesis of all results is given and the mechanisms of bidirectional communication between the neuroendocrine and the immune system are discussed in the light of future research. An emphasis is put on the significance of glucocorticoid receptors not merely in stress but also in immune regulation.

'You can't learn a science unless you know what it's all about.'

Aldous Huxley, Brave New World

2

Evolution of glucocorticoid receptors with different glucocorticoid sensitivity

Ellen H. Stolte, B.M. Lidy Verburg-van Kemenade, Huub F.J. Savelkoul, Gert Flik

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Abstract

Glucocorticoids (GCs) are commonly used to treat a variety of immune diseases. However, the efficacy of treatment is greatly influenced by an individual variation in sensitivity to GCs, which is caused by differences in the glucocorticoid receptor (GR). The variable receptor profile results from variations in the GR gene, or alternative splicing of the gene coded. We investigated the evolution of the GR gene by comparing genomic GR sequences of vertebrates. Exon length and amino acid sequence are conserved among all classes of vertebrates studied, which indicates strong evolutionary pressure on conservation of this gene. Interestingly, teleostean fishes have two different GR proteins. One of the duplicate fish GR genes has a nine amino acid insert in the DNA binding region, that results from alternative splicing. The duplicate GR genes and products of alternative splicing in teleostean fishes are differentially expressed *in vivo* and show different transactivation capacity *in vitro*. The presence of two GR genes appears to be a result of divergence of receptors rather than of ligands. Teleostean fishes express different, evolutionary related, functional GR proteins within a single organism. Hereby teleostean fishes present a model that facilitates investigation of the molecular basis of cortisol resistance and different regulatory functions of cortisol.

2.1 Introduction

Glucocorticoids (GCs) (cortisol and corticosterone) play a pivotal role in vertebrate physiology through a plethora of control mechanisms. The glucocorticoid system, with a nuclear glucocorticoid receptor (GR) is found in all vertebrates, consistent with an evolutionary well-conserved stress response. GCs regulate cell-growth, bone density, metabolism, cardiovascular system and influence behaviour (Charmandari *et al.*, 2005). Moreover, *in vitro* studies with human, murine and rat immune cells showed the immunosuppressive effects of elevated GC levels, following stressful circumstances. GCs suppress Th1 cellular immunity and mediate a Th2 shift by suppressing production of Th1 (TNF-α, IFN-γ, IL-2, and IL-12) cytokines and inducing production of Th2 (IL-4, IL-10 and IL-13) cytokines (Elenkov, 2004).

For this reason, GCs are used to treat a variety of immune diseases. Local anti-inflammatory properties of GCs make it a first choice medication for asthma (Walsh, 2005) and rheumatoid arthritis (Boers, 2004). However, there is considerable individual variation in sensitivity to GCs (Hearing *et al.*, 1999), which may affect the outcome of GC-treatment. Mutations and splice variation in the GR gene and thus polymorphisms in the protein in humans and other vertebrates may explain the individual variation in sensitivity rather than alteration in the consensus sequence of the GR binding site in the DNA of target genes (Brandon *et al.*, 1991; Bray and Cotton, 2003; DeRijk *et al.*, 2002; Keightley and Fuller, 1994; Lamberts *et al.*, 1996; Stevens *et al.*, 2004b; van Rossum *et al.*, 2002).

Recently it was shown that two teleostean (bony) fishes, the rainbow trout (*Oncorhynchus mykiss*; (Bury *et al.*, 2003)) and Burton's mouthbrooder (*Haplochromis burtoni*; (Greenwood *et al.*, 2003)), each have two different GR genes. The two different receptors encoded by these genes display high amino acid sequence identity especially in the part of the gene coding for the C-terminal part of the receptor protein. Rainbow trout GR1 and GR2 show 51 % overall identity, and 86 % identity in the C-terminal part (DNA binding region, hinge region and ligand binding region), for Burton's mouthbrooder, these percentages are 49 % and 80 % respectively. Moreover, alternative splicing occurs in these fish GR genes (Greenwood *et al.*, 2003; Takeo *et al.*, 1996), which leads to functional transcripts with different transactivation properties compared to 'wildtype'. Both the duplicate GR genes as well as the splice variants of these GR genes in fish are differentially expressed in tissues and differ in their affinity for cortisol, the single dominant glucocorticoid in fish (Bury *et al.*, 2003; Greenwood *et al.*, 2003).

We question whether differences among GRs affect the sensitivity to GCs and the po-

tential impact of these differences for biological responses. We address the meaning of duplicate genes and splice variants. Genomic data base information and review of the literature give evidence that duplicate GR genes and alternative splicing are common features of all teleostean fishes and set them apart from other vertebrate taxa.

2.2 Glucocorticoid receptor

The glucocorticoid receptor (GR) (Hollenberg *et al.*, 1985) structure and function is well established in mammals and conserved among all vertebrate species analysed so far. The GR belongs to the nuclear receptor super family (Evans, 2005; Fuller, 1991; Kumar and Thompson, 1999; Mangelsdorf *et al.*, 1995). Its members act as ligand-dependent transcription factors. All receptors in this superfamily, which include those for steroid hormones, thyroid hormones, retinoic acid and vitamin D_3, share a similar domain structure, which was first predicted for the GR (Giguere *et al.*, 1986) (figure 2.1).

The N-terminal region varies greatly among different members of the superfamily, both in size and composition, and is involved in activation of down stream genes (transactivation). Mutations in this domain decrease transcriptional activity of target genes without affecting ligand affinity (Giguere *et al.*, 1986). The DNA-binding region (see below) is the central domain and binds to glucocorticoid responsive elements (GRE) in promoter regions to initiate transcription of a vast array of GC responsive genes. The amino acid sequence of this

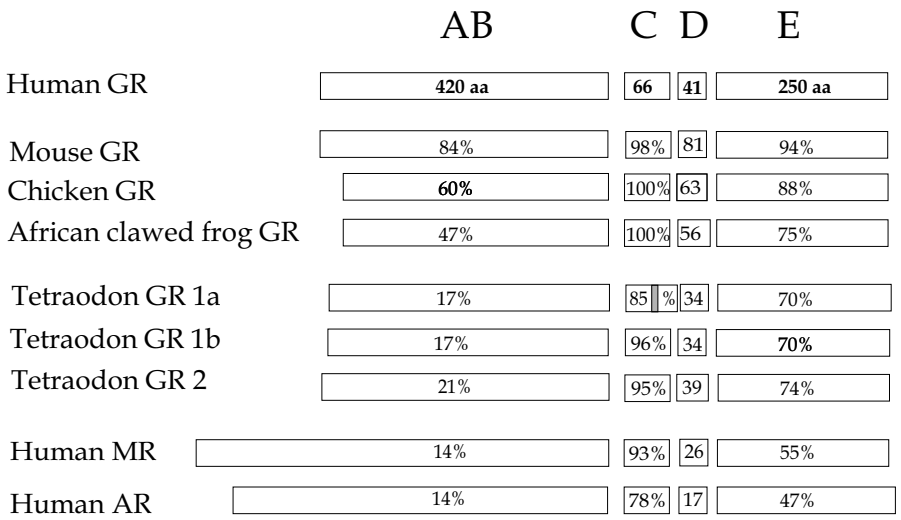

Figure 2.1: Percentage amino acid identity of the different receptor regions of several members of the nuclear receptor superfamily of vertebrate species. Length of the boxes represents lengths of particular regions. MR; mineralocorticoid receptor, AR; androgen receptor, AB; N-terminal region, C; DNA binding region, D; hinge region, E; ligand binding region.

region is strictly conserved, both in different members of the superfamily and in virtually all vertebrate species (figure 2.1). The hinge region, involved in conformational changes during receptor-ligand binding, is quite variable in its length and amino acid sequence. The relatively well-conserved ligand binding region (see below) is situated at the C-terminus. The marked similarity of amino acid sequences in the ligand binding region explains why multiple receptors can bind the same ligand and subsequently elicit a similar response (activation of a downstream gene) in an *in vitro* transactivation assay. In these assays a cell lacking endogenous corticoid receptors is co-transfected with a transcription vector with the corticoid receptor to be tested and a reporter plasmid bearing one or multiple GREs in its promoter. After addition of the ligand, the *in vitro* expressed corticoid receptor protein can bind the ligand and translocate to the nucleus where it will subsequently bind to the GRE on the promoter of the reporter gene and induce transcription of this gene. These *in vitro* experiments showed that cortisol enhanced transcriptional activity of the mineralocorticoid receptor (MR) of Burton's mouthbrooder (EC_{50} 0.02 nM) at a lower concentration than that required for transactivation via the GR (EC_{50} 2–5 nM) in one and the same assay. In rainbow trout a similar result was found; the MR required a lower concentration of cortisol to induce transcriptional activity and a difference was seen for the duplicate GRs with EC_{50}s of ≈ 100 nM, ≈ 10 nM and ≈ 1 nM, for GR1, GR2 and MR, respectively (Greenwood *et al.*, 2003; Sturm *et al.*, 2005).

2.2.1 DNA binding region

The 3D-structure of the DNA binding region of the human GR was confirmed by nuclear magnetic resonance imaging (NMR) and it shows two sub-domains (CI and CII), each consisting of a zinc finger (Hard *et al.*, 1990).

The most N-terminally located zinc finger is involved in binding of the receptor to DNA. Site specific DNA recognition depends on amino acids GSV in the P (proximal) box that bind to the hormone responsive element (GRE) of target genes (Luisi *et al.*, 1991; Umesono and Evans, 1989). The main loop of this N-terminal zinc finger is a short segment of antiparallel β-sheet, whereas the second pair of cysteines is at the start of an α-helix that provides the contact with the major groove of the GRE. This α-helix between Ser^{459} and Glu^{469}(in rat) is referred to as the DNA recognition helix (Hard *et al.*, 1990; Luisi *et al.*, 1991). The more C-terminal zinc finger is involved in receptor homo-dimerisation at the GRE and stabilises binding of the GR to the GRE, which is mediated by amino acids AGRND in the D (distal) box (Kumar and Thompson, 1999, 2005; Luisi *et al.*, 1991). The zinc fingers together with the conserved amino acids in the two sub-domains result

in the 3D structure required for specific DNA binding and thereby for the downstream effects of GCs. This is demonstrated by insertional mutagenesis, where disruption of the C-x-x-C motif silences transcriptional activity of the receptor without interfering with the ligand-binding ability (Giguere *et al.*, 1986; Wickert and Selbig, 2002).

2.2.2 Ligand binding region and heat shock proteins (Hsps)

To allow binding of the ligand, the receptor conformation has to change, to give the ligand access to a hydrophobic cleft in the ligand binding region (Pratt and Toft, 2003). Heat shock proteins Hsp90 and Hsp70 are both essential as chaperones for the opening of this cleft (Morishima *et al.*, 2000b). In formation of the hormone receptor complex the chaperone Hsp70 first binds directly to the ligand binding region, which induces a conformational change in the GR that initiates the opening of the steroid binding cleft, an ATP consuming process (Morishima *et al.*, 2000a). Hsp90 binds to this primed GR*HSp70 complex and completely opens the cleft. As soon as the steroid binding cleft is opened, cortisol enters and binds to the hydrophobic pocket of the ligand binding region (Kanelakis *et al.*, 2002; Morishima *et al.*, 2000a; Pratt and Toft, 2003). After binding of the hormone with the GR*Hsp70*Hsp90 (GR-hetero) complex, the chaperone complex dissociates. The receptor conformation returns from a labile open structure into a stable compact DNA binding state that can exist independently of chaperone complexes (Pratt and Toft, 1997). Subsequently the receptor is translocated into the nucleus where it will homodimerise and bind as a homodimer to the specific DNA motifs (GREs) which in turn will activate transcription of the down-stream genes (Schoneveld *et al.*, 2004).

However, translocation of the receptor to the nucleus depends on phosphorylation of the receptor (Ismaili and Garabedian, 2004). Furthermore, phosphorylation is involved in the half-life of GR and in downregulation following ligand treatment (Webster *et al.*, 1997). Thus, GR phosphorylation has an essential effect on the ultimate receptor function (Ismaili and Garabedian, 2004).

Although details of the formation of the GR-heterocomplex are yet to be established in teleostean fishes, we assume, based on sequence conservation of Hsp70, Hsp90 and the GR ligand binding region, that comparable processes take place in all fish and thus in vertebrates. Currently, data on phosphorylation of the teleostean fish GR proteins are not available. However, we will make a prediction based on conserved phosphorylation consensus motifs in alignments of the N-terminal region of fish and mammalian sequences.

2.3 Stress and the immune system

The stress response of all vertebrates, including teleostean fishes, is comparable. In fish, stress signals are conveyed from the hypothalamus via corticotropin-releasing hormone (CRH) to the pituitary, where adrenocorticotropic hormone (ACTH) is released into the circulation. ACTH in turn activates the cortisol producing cells of the head kidney, the functional equivalent of the adrenal gland. In fish under acute stress, cortisol levels can easily increase more than ten-fold, from 10–20 ng/ml to over 350 ng/ml (Wendelaar Bonga, 1997). Both basal levels as well as increases in cortisol level during stress are in the same range as those in the mammalian stress response (Hennessy *et al.*, 1997; Piekarzewska *et al.*, 2000; Van Cauter *et al.*, 1996).

High cortisol levels significantly affect the immune system (Charmandari *et al.*, 2005; Franchimont, 2004; Sapolsky *et al.*, 2000). However, the resulting response depends on the parameter studied and type of immune cell involved. *In vitro* studies showed that in common carp high levels of cortisol, as occur during stress, rescue neutrophilic granulocytes from apoptosis by neglect (Weyts *et al.*, 1998a). Activated B-lymphocytes however, show increased apoptosis when exposed to high levels of cortisol (Weyts *et al.*, 1998c). Thus, in stressed fish the innate immune system becomes activated. This is further illustrated by the redistribution of neutrophilic granulocytes from the head kidney into circulation after immersion vaccination (Huising *et al.*, 2003a). This stress-induced neutrophilia is considered to improve peripheral surveillance and is also reported in mammals (Dhabhar *et al.*, 1996). Meanwhile, the adaptive immune system becomes temporarily repressed (Franchimont, 2004).

2.4 Evolution of vertebrate GRs

GR proteins of teleostean fishes and other vertebrates were compared based on genomic sequences and gene structure of representatives of mammals, birds, amphibians and bony fishes. Moreover, the duplicate amino acid sequences of Burton's mouthbrooder and rainbow trout GR proteins and other fish species for which to date only a single GR protein has been identified, were investigated. From this information we dare to make general predictions about the similarities and, more interestingly, the differences of fish GR sequences compared to other vertebrate GR sequences.

In this review we obey the present rainbow trout GR nomenclature with subscripts **a** and **b** for splice variants (Bury *et al.*, 2003). In this nomenclature GR1 has alternative

splicing; in which GR1a has a nine-amino-acid insert and GR1b does not. In GR2 alternative splicing has so far not been reported. Unfortunately, the published nomenclature of Burton's mouthbrooder is deviant; GR2b has the nine-amino-acid insert as a result of alternative splicing, and GR2a does not have this insert. The GR1 gene does not show alternative splicing (Greenwood *et al.*, 2003).

2.4.1 Conservation of sequence identity, but two different GR genes

There are over 23.000 species of teleostean fishes, compared to 'only' 14.000 species of birds and mammals taken together (Nelson, 1994). Species of teleostean fishes are vastly different and live in very diverse ecosystems, but still use the same basic regulatory mechanisms.

As mentioned above, it has been shown that both rainbow trout as well as Burton's mouthbrooder have duplicated GR genes (Bury *et al.*, 2003; Greenwood *et al.*, 2003). It was then investigated whether other teleostean fishes also have duplicate copies of their GR gene. Using the Ensembl genome database, the gene structures of both tetraodon (*Tetraodon nigroviridis*) and fugu (*Takifugu rubripes*) GR genes were predicted. Both these teleostean fishes were considered ideal for sequencing as their genomes are extremely compact due to a major lack of intronic DNA and this enabled efficient comparisons with other vertebrate genomes (Hedges and Kumar, 2002). As predicted, in the genome of both these species duplicate copies of the GR gene were found. These two different proteins are encoded on different scaffolds in fugu and on different chromosomes in tetraodon (GR1 on chromosome 7 and GR2 on chromosome 1). The duplicate copies of the GR genes have conserved amino acid sequences but are distinctly different. Both teleostean fish GR genes are conserved in exon length (figure 2.3) and amino acid sequences (figure 2.1). The zebrafish (*Danio rerio*) genome also has been sequenced. To date however, only one zebrafish GR gene has been identified.

A phylogenetic tree was constructed, comparing the GR sequences of different vertebrate species using the neighbour-joining method (Kumar *et al.*, 2004; Saitou and Nei, 1987) (figure 2.2).

This phylogenetic tree clearly shows a cluster of tetrapod GRs and a cluster of teleostean fish GRs. This cluster of teleostean GRs is subdivided into two different clades, representing two different GR genes. The duplicate GR genes of teleostean fishes appear to be the result of the genome duplication that occurred early in the evolution of actinopterygii, *i.e.* before the radiation of the teleostean fishes, and after the divergence of tetrapods from the fish lineages, which occurred around 450 million years ago (Hedges and Kumar, 2002). Duplicate GR genes are seen in both salmonids (rainbow trout) and percomorphs (fugu,

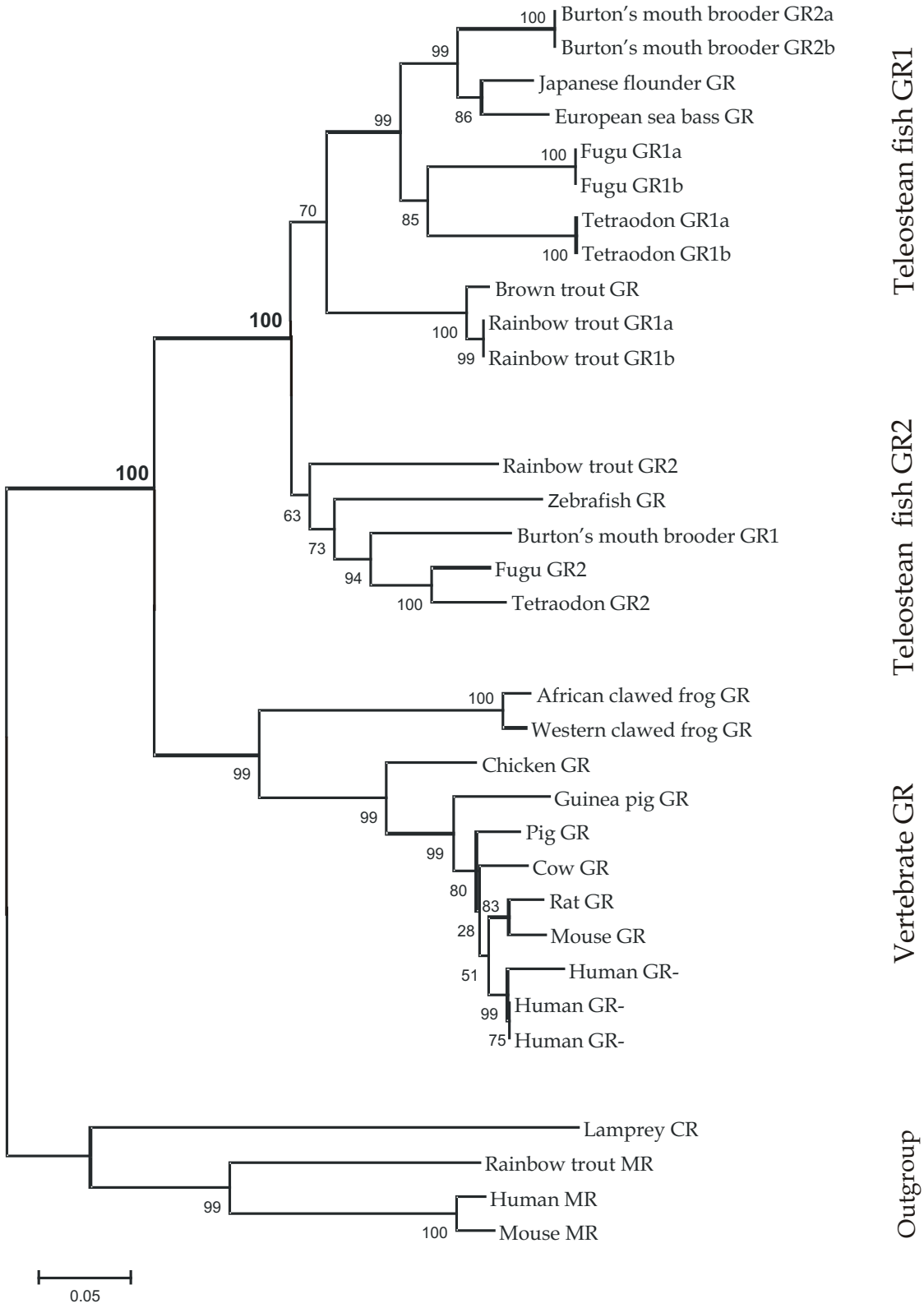
Burton's mouth brooder GR2a
Burton's mouth brooder GR2b
Japanese flounder GR
European sea bass GR
Fugu GR1a
Fugu GR1b
Tetraodon GR1a
Tetraodon GR1b
Brown trout GR
Rainbow trout GR1a
Rainbow trout GR1b
Rainbow trout GR2
Zebrafish GR
Burton's mouth brooder GR1
Fugu GR2
Tetraodon GR2
African clawed frog GR
Western clawed frog GR
Chicken GR
Guinea pig GR
Pig GR
Cow GR
Rat GR
Mouse GR
Human GR-
Human GR-
Human GR-
Lamprey CR
Rainbow trout MR
Human MR
Mouse MR
0.05
Teleostean fish GR1
Teleostean fish GR2
Vertebrate GR
Outgroup

Figure 2.2: (*previous page*) Phylogenetic tree; comparison of the amino acid sequences of the vertebrate glucocorticoid receptors (GRs) and corticoid receptor (CR). This tree was generated with MEGA version 3.1 software using the neighbour-joining method. Reliability of this tree was assessed by bootstrapping using 1000 bootstrap replications (values in percentage are indicated at branch nodes). Mineralocorticoid receptor (MR) sequences were used as outgroup. Human (*Homo sapiens*) GR-α; P04150, Human GR-β; NP_001018661, Human GR-γ; NP_001019265, Mouse (*Mus musculus*) GR; P06537, Rat (*Rattus norvegicus*) GR; NP_036708, Guinea pig (*Cavia porcellus*) GR; P49115, Pig (*Sus scrofa*) GR; AY779185, Cow (*Bos taurus*) GR; AY238475, Chicken (*Gallus gallus*) GR; Q8JHA4, African clawed frog (*Xenopus laevis*) GR; P49844, Western clawed frog (*Xenopus tropicalis*) GR; NM_001016967, Zebrafish (*Danio rerio*) GR; ENSDARESTT00000005443 (Chr. 14), Rainbow trout (*Oncorhynchus mykiss*) GR1; P49843, GR2; AY4953720, Burton's mouth brooder (*Haplochromis burtoni*); GR1; AF263738, GR2a; AF263739, GR2b; AF263740, Japanese flounder (*Paralichthys olivaceus*) GR; O73673, European sea bass (*Dicentrarchus labrax*) GR; AY549305, Brown trout (*Salmo trutta*) GR; AY863149, Sea lamprey (*Petromyzon marinus*) (ancestral) CR; AY028457, Human MR; M16801, Mouse MR; XP_356093, Rainbow trout MR; AY495584

tetraodon, Burton's mouthbrooder), that are two lineages of fish species that diverted very early in teleostean fish evolution. Therefore it is reasonable to assume that the presence of duplicate GR genes throughout fish species relates to the fish-specific genome duplication that occurred approximately 300 million years ago (Hoegg *et al.*, 2004; Nelson, 1994; Volff, 2005).

A 'corticoid receptor' of a sea lamprey (*Petromyzon marinus*) was included in our analysis. This receptor was named 'corticoid receptor' as it resembled both the vertebrate corticoid receptors MR and GR. Sea lamprey is an agnathan (jawless) fish of an evolutionary lineage that preceded the teleostean fish lineage. Moreover, it was predicted that evolution of the vertebrate steroid receptors took place from an ancestral gene through serial genome expansions. In this hypothesis, a common corticoid receptor gene was the ancestral entity for both the GR and the MR (Thornton, 2001). Indeed, our analysis shows that the lamprey corticoid receptor seems to cluster with the outgroup MR, not with any of the GR genes. Based on this phylogenetic tree we therefore cannot predict which of the duplicate GR genes is more reminiscent of the ancestral GR.

If the duplication of the GR gene is indeed a result of the fish-specific genome duplication, we predict that many more teleostean fishes will have duplicate copies of their GR gene. In fact, from the genome point of view, all teleostean fishes should have at least duplicate GR genes, but one of the copies could of course be secondarily lost due to mutational events if one copy was sufficient for biological function.

Table 2.1: Putative phosphorylation motifs in the glucocorticoid receptors of different species. Depicted are the residues phosphorylated in mouse GR and a score if these residues are conserved in mammals, chicken, African clawed frog or teleostean fishes. The consensus motif for a particular phosphorylation site is mentioned for mammals and teleostean fishes separately. The putative protein kinases expected to target these motifs are also shown separately and are based on mammalian literature. This table was constructed after analysis of a multiple sequence alignment of Human (*Homo sapiens*) GR-α; P04150, Mouse (*Mus musculus*) GR; P06537, Rat (*Rattus norvegicus*) GR; NP_036708, Guinea pig (*Cavia porcellus*) GR; P49115, Pig (*Sus scrofa*) GR; AY779185, Cow (*Bos taurus*) GR; AY238475, Chicken (*Gallus gallus*) GR; Q8JHA4, African clawed frog (*Xenopus laevis*) GR; P49844, Zebrafish (*Danio rerio*) GR; ENSDARESTT00000005443 (Chr. 14), Rainbow trout (*Oncorhynchus mykiss*) GR1; P49843, GR2; AY4953720, Burton's mouth brooder (*Haplochromis burtoni*) GR1; AF263738, GR2b; AF263740, Japanese flounder (*Paralichthys olivaceus*) GR; O73673, European sea bass (*Dicentrarchus labrax*) GR; AY549305, Brown trout (*Salmo trutta*) GR; AY863149, Fugu (*Takifugu rubripes*) GR1; GENSCAN00000003615 (scaffold 1264) & GENSCAN00000029451 (scaffold 4328), GR2; SINFRUG00000143550 (scaffold 59), Tetraodon (*Tetraodon nigroviridis*) GR1; GIDT00024792001 (Chr. 7), GR2; GSTENG00017027001 (Chr. 1).

(a) Mouse residue scores in other vertebrates.

Mouse	Mammals	Chicken	African clawed frog	Teleostean fishes
S122	Not in guinea pig	Yes	Yes	No
S150	Not in guinea pig	No	No	No
T171	Only in rat	No	No	No
S212	All	Yes	Different	Different
S220	Not in pig, not in cow	Yes	No	No
S234	All	Yes	Yes	No
S315	Not in human	Yes	Yes	Yes

(b) Consensus motifs and putative protein kinases.

Mouse	Consensus mammals	Alternative consensus	Kinases mammals	Alternative kinases
S122	S/T(P)-x-x-E/D		CKII	
S150	None			
T171	Non polar-x-S/T(P)		MAPK/GSK3	
S212	S/T(P)-x-R/K		CDK	
S220	S/T(P)-x-R/K	S/T(P)-x-x-E/D	CDK	CKII
S234	Non polar-x-S/T(P)		MAPK	
S315	None			

2.4.2 Phosphorylation sites in N-terminal region of fish GR

As mentioned above, phosphorylation has significant effects on GR function. By comparing the mouse, human and rat phosphorylation sites Ismaili and coworkers constructed an instructive scheme of consensus sequences for phosphorylation and the putative protein kinases that are predicted to target certain motifs (Ismaili and Garabedian, 2004). By alignment of the GR gene sequence of several vertebrate species (both mammalian and fish) predictions can be made about presence and number of phosphorylation sites in teleostean fishes (table 2.1).

The mammals in this alignment show good similarity. Similarity decreased when chicken, African clawed frog and teleostean fishes were compared. The serine residues corresponding to Ser^{122} and Ser^{234} in mouse are conserved in mammals, chicken and African clawed frog. The serine corresponding to Ser^{315} in mouse is conserved throughout all the species but as there is no consensus motif it is hard to predict if this serine is indeed part of a true phosphorylation site in all the species.

The alignment suggests that the consensus motif for the casein kinase II, (CKII; S/T(P)-x-x-E/D) is widely present in the AB region of teleostean fishes and found at conserved locations in a series of fish species. Moreover, all fish species examined show one or more mitogen-activated protein kinases (MAPK) motifs (non-polar-x-S/T(P)-P) in their AB domain, yet these motifs, do not correspond to conserved locations. Furthermore, the consensus motif for CDK (S/T(P)-P-x-R/K) could not be found in any of the fish species examined. This indicates that fish exploit a different consensus motif for the cyclin dependent kinases (CDKs), or that different kinases are used to phosphorylate the GR. Indeed, at the location corresponding to Ser^{212} in mouse the fish species have a different consensus motif (S/T(P)-x-x-E/D) than mammals.

Whether the conserved serines and conserved consensus motifs for the different kinases do indeed correspond with phosphorylation sites needs to be confirmed by peptide mapping, mutagenesis, and phosphorylation studies.

2.4.3 DNA binding region in fish GR

Another interesting difference between GR proteins in teleostean fishes and other vertebrates is seen in the DNA binding region. This C-domain is the best conserved region of the GR protein (98 %, 100 %, 100 %, 85 % amino acid identity between human GR and mouse, chicken, xenopus and tetraodon GR1a sequences, respectively). However, some fish GR1 genes show a 27 nucleotide insert that encodes nine additional amino acids. Apart

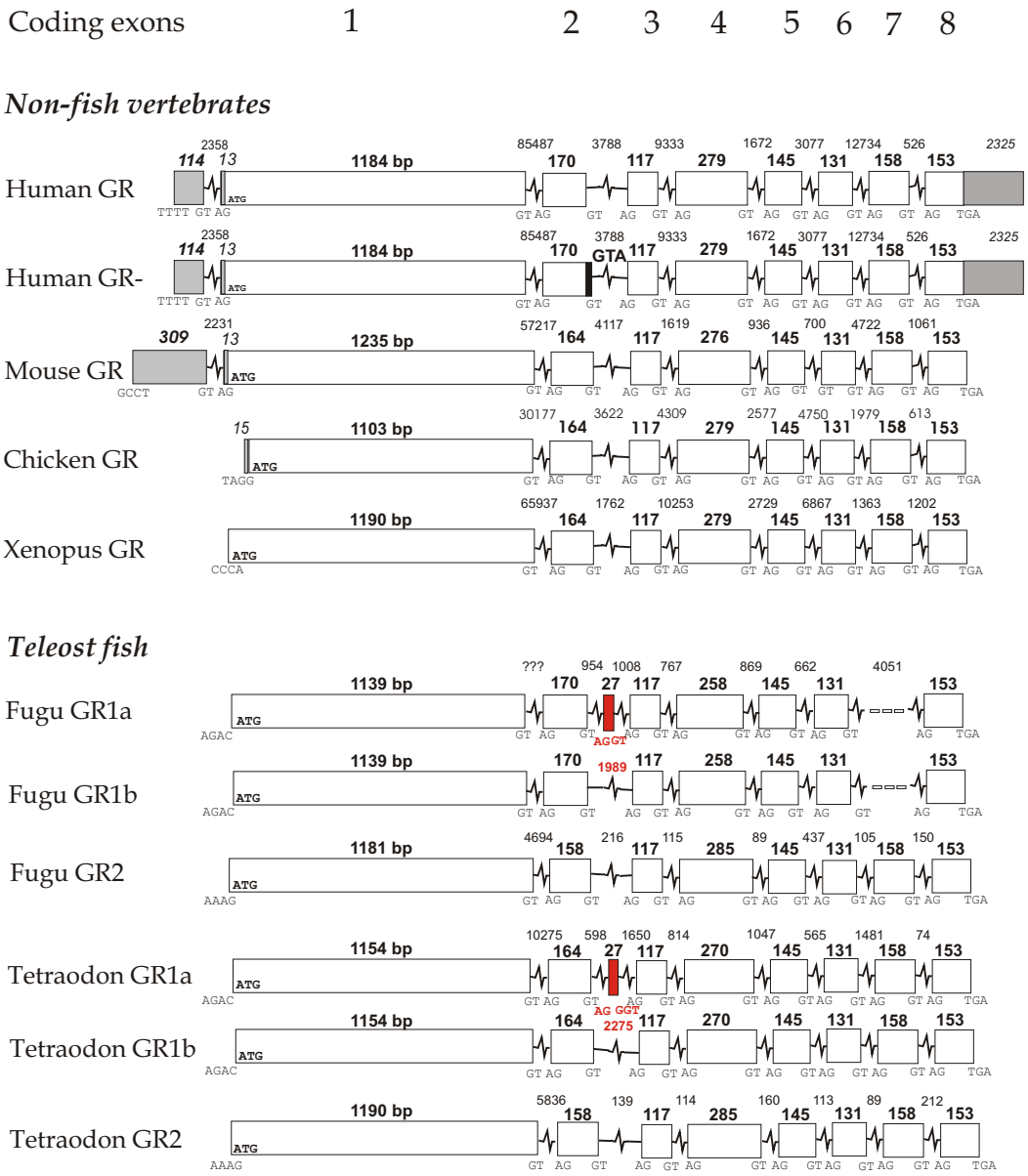

Figure 2.3: Gene structure of vertebrate GR receptors. Exon lengths are shown in bold and intron lengths in normal cases. Splice sequences flanking the exons and nucleotide sequence preceding start codon are shown above blocks representing exons. Untranslated (parts of) exons are depicted in light grey. Elongation of exon 3 by alternative splicing in human GR-γ is shown in dark grey. Extra exon, only present in splice variants in fish GR is depicted in black. Exon 8 in Fugu GR1 could not be found in the Ensembl database. Human (*Homo sapiens*) GR; ENSG00000113580, Mouse (*Mus musculus*) GR; ENSMUSG00000024431, Chicken (*Gallus gallus*) GR; ENSGALG00000007394, Western clawed frog (*Xenopus tropicalis*) GR; ENSXETT00000003968, Fugu (*Takifugu rubripes*) GR1; GENSCAN00000003615 (scaffold 1264) & GENSCAN00000029451 (scaffold 4328), GR2; SINFRUG00000143550 (scaffold 59), Tetraodon (*Tetraodon nigroviridis*) GR1; GIDT00024792001 (Chr. 7), GR2; GSTENG00017027001 (Chr. 1).

from this insert the C-domain of teleostean fishes is almost identical to that in other vertebrates: 96 % vs 85 % amino acid identity between human GR and tetraodon GR1b and GR1a, respectively) (figure 2.1). These nine amino acid inserts between the two zinc fingers are also remarkably conserved among teleostean fish species. In rainbow trout (Ducouret *et al.*, 1995; Takeo *et al.*, 1996), Japanese eel (*Anguilla japonica*) (Todo and Nagahama, 1998), Japanese flounder (*Paralichthys olivaceus)* (Tokuda *et al.*, 1999), Burton's mouthbrooder (Greenwood *et al.*, 2003), brown trout (*Salmo trutta*; AY863149), European sea bass (*Dicentrarchus labrax*; AY549305) and fugu (*Takifugu rubripes*) a **WRARQNTDG** insert is present. A slightly different insert is found in tetraodon (*Tetraodon nigroviridis)*: **WRARQNTVC**. Especially the D to V substitution is interesting, as aspartic acid is hydrophilic and negatively charged, whereas valine is hydrophobic. Glycine and cysteine are also substantially different with regard to size and biochemical properties.

The nine amino acid inserts appear to be the result of alternative splicing and are encoded by an extra exon. This extra exon is 'hidden' in the intron that separates exons three and four (figure 2.3). This is evident from the genomic sequences of fugu and tetraodon and was confirmed experimentally by the amplification of a genomic DNA fragment in rainbow trout (Lethimonier *et al.*, 2002).

2.4.4 Duplicate GRs; expression and transactivation capacities

The presence of duplicate GR genes which also show this particular alternative splicing gives reason to hypothesise that the resulting duplicate receptor proteins have separated functions. This hypothesis is strengthened as teleostean fishes do not possess the mineralocorticoid aldosterone and use cortisol instead for osmoregulation (Dean *et al.*, 2003; Metz *et al.*, 2003). This adds another important function to the long list of different functions to be regulated by one hormone. To be able to regulate all these different functions with a single hormone it is likely to assume that differentiation occurred in the hormone receptor.

Differences in receptor protein function could be indicated by differential expression of the duplicate GR genes in different organs. A literature comparison of different species of fish reveals that both genes are expressed in all organs examined. In all these species GR1 (the gene showing the nine amino acid insert) is more strongly expressed than the GR2 gene (Bury *et al.*, 2003; Greenwood *et al.*, 2003). Moreover, most first reported teleostean fish GR sequences, have the nine amino acid insert and would therefore be classified as GR1, such as in European sea bass and brown trout (Ducouret *et al.*, 1995; Takeo *et al.*, 1996; Todo and Nagahama, 1998; Tokuda *et al.*, 1999). Whether this reflects relative abundance of the GR1 messenger, or preferential primer design is unclear.

Separate biological functions of the duplicate GR forms may further result from different affinity for the ligand or from different transactivation properties. Information about ligand binding is scarce, but in rainbow trout it was reported that duplicate GR genes have similar dexamethasone binding affinities that are comparable to the affinity of human GR for dexamethasone (Bury *et al.*, 2003; Ducouret *et al.*, 1995; Ray *et al.*, 1999). However, the resulting biological effect as measured with transactivation assays is quite different for the duplicate GR genes.

In rainbow trout concentrations of cortisol required to induce activation of downstream genes is significantly different between the duplicate GR proteins. Rainbow trout (rt)GR2 requires lower concentrations of cortisol (EC_{50} of 0.7 nM), than rtGR1a (with nine amino acid insert; EC_{50} of 46 nM) to induce transactivation (Bury *et al.*, 2003).

Variation in transactivation properties of the receptor after stimulation with a ligand is not only the result of the GR protein involved. Variation in transactivation also depends greatly on the ligand used. In *in vitro* experiments in rainbow trout the GR agonist dexamethasone activated the tested downstream gene at lower concentrations than cortisol in the same experiment; EC_{50} for GR1 stimulated with cortisol was 46 nM while it was 4.3 nM with dexamethasone treatment, for GR2 this was 0.7 nM for cortisol and 0.35 nM for dexamethasone (Bury *et al.*, 2003). In other experiments it was shown that with the same concentration of ligand, dexamethasone gave a stronger activation of the reporter gene than cortisol (Greenwood *et al.*, 2003; Takeo *et al.*, 1996; Tokuda *et al.*, 1999).

It is crucial to keep this difference between cortisol and dexamethasone in mind when testing newly found GR genes or GR variants. Indeed, experiments with dexamethasone might slightly (rainbow trout GR2; twofold) or severely (rainbow trout GR1; tenfold) overestimate the cortisol-induced transactivation capacity.

2.4.5 GR splice variants; expression and transactivation capacities

Several different splice variants are described for the GR gene (Geng *et al.*, 2005; Oakley *et al.*, 1999). However, in this review we focus only on the splice variations in the DNA binding region of the GR gene.

Again as with the duplicate genes, it is interesting to hypothesise about functional differences between the two splice variants. The expression level of the GR1 variant with the nine amino acid insert exceeds that of the non-spliced form (Bury *et al.*, 2003; Greenwood *et al.*, 2003; Takeo *et al.*, 1996). In Burton's mouthbrooder the variant without the insert was found in all organs tested, in rainbow trout however, it could only be detected in testis (Greenwood *et al.*, 2003; Takeo *et al.*, 1996). As mentioned before, most teleostean fish GR

sequences that were found show this nine amino acid insert (Ducouret *et al.*, 1995; Takeo *et al.*, 1996; Todo and Nagahama, 1998; Tokuda *et al.*, 1999). It remains to be investigated if in all species mentioned a variant of the GR1 gene without the 9 amino acid insert is also expressed. This variant can easily be overlooked as the difference between the two is so small that specific primers are required to detect them. Differences in biological functions of the splice variants could also result from different affinity for the ligand or different transactivation properties related to this difference in affinity. As there is no difference between the splice variants in the ligand-binding region it is unlikely that there is any effect on ligand binding affinity.

The only differences between the splice variants are found in the DNA-binding region and indeed, differences in DNA-binding affinity do occur. In an experiment with rainbow trout two different promoters were used. One luciferase reporter plasmid contained one and the other two GRE elements from the rat tyrosine aminotransferase promoter. Interestingly, whereas the GR1a (with the nine amino acid insert) has a better binding affinity for single GRE than the GR1b, the opposite was true for the double GRE sequence (Lethimonier *et al.*, 2002).

Differences in the final transactivation capacity also occur. QUANTA Protein Design computer predictions of corticoid receptor DNA binding domain function based on secondary structure analysis showed that insertions of one, four, or nine amino acids residues are possible without destruction of the secondary structure. However, in all models predicted, the glutamic acid Glu^{469} (in rat) was no longer included in forming the DNA recognition helix. The nine amino acid insert seen in teleostean fishes may form a loop that extends to the outside of the protein. Receptor function predicted by homology modelling of this peculiar receptor did not differ from functioning of wild-type receptor (Wickert and Selbig, 2002). A Predict Protein software analysis predicts that the nine amino acid insert would lengthen the α-helix involved in GRE recognition (Lethimonier *et al.*, 2002). In rainbow trout, it was experimentally shown *in vitro*, that transactivation after stimulation of rtGR1a (with insert) with dexamethasone or cortisol was increased compared to GR1b (without the insert). These results were obtained by the use of RBCF-1 cells that are derived from goldfish fin tissue and a MSG-cat reporter plasmid (Takeo *et al.*, 1996). In another experiment with rainbow trout GR using Chinese hamster ovary (CHO-K1) cells it was again shown that the use of a single GRE luciferase reporter plasmid the GR1a gave a stronger hormone-independent transactivation than GR1b. However, with a double GRE reporter gene, this difference was abolished (Lethimonier *et al.*, 2002). Moreover, in an *in vitro* co-transfection of Burton's mouthbrooder with a triple GRE in the tat3-luciferase reporter plasmid, GR2b

(with nine-amino-acid insert) showed only half maximal activation in response to cortisol compared to GR2a (without insert). These experiments were carried out with CV1b (corticoid receptor-deficient primate renal) cells (Greenwood *et al.*, 2003). Differences between triple, double and single GRE promoters therefore seem to substantially contribute to the results obtained and hamper a good comparison.

Receptor functionality has been shown for the GR splice variant with the nine amino acid insert in rainbow trout, Burton's mouthbrooder and Japanese flounder (Tokuda *et al.*, 1999). Therefore it is assumed that other teleostean GR proteins, with this particular nine amino acid insert (or a slight variation thereof) at exactly the same location in the DNA binding region, will also be functionally active.

Alternative splicing has been reported in mammalian species as well. Human small lung cell carcinoma exhibits a GC-resistant phenotype, which was thought to be caused by a tri-nucleotide insertion in the GR gene; this insertion translates into an additional amino acid, Arg^{453} (Ray *et al.*, 1996). This particular human GR splice variant was later named hGR-γ (Rivers *et al.*, 1999). Although the insertion found in hGR-γ occurs at exactly the same location as the nine amino acid insert in the fish GR, it results from a different splice variation process. In the human GR-γ an alternative splice site is used that is located three nucleotides downstream of the original splice site at the 3' end of coding exon 3. This leads to an insertion of one extra codon in exon 3 (figure 2.3) (Rivers *et al.*, 1999). In teleostean fishes, in contrast, the original splice site of exon 3 is used, and a whole new exon is introduced between exons 3 and 4. The intron in between exons 3 and 4 contains this extra 27 nucleotide exon, which is flanked by common splice sequences AG at the 5' end and GT at the 3' end (figure 2.3). Human GR-γ is ubiquitously expressed, but only accounts for 5 % of all GR transcripts (Rivers *et al.*, 1999; Stevens *et al.*, 2004a).

The addition of an arginine as in the human GR-γ or the marmoset monkey (see below) would create a new hydrogen bond and therefore allow a new 3-turn to be formed, which could affect affinity of receptor-DNA binding (Wickert and Selbig, 2002). Indeed, the human GR-γ (Arg^{453}) is half maximally activated compared to wild-type with a similar EC_{50} in an *in vitro* co-transfection assay with GR Arg^{453} and MMTV-luciferase reporter plasmid in COS7 (African green monkey kidney) cells (Ray *et al.*, 1996). Alternative splicing comparable to that in human GR-γ is also seen in the cotton-top marmoset (*Saguinus oedipus*) and thus is of wider occurrence (Brandon *et al.*, 1991).

Collectively these results show that the specificity of the alternatively spliced GRs for the specific target genes tested seems unaffected, but the differences in affinity of DNA binding or efficiency of dimerisation result in changes in transactivation capacity of the GR1 protein

with the nine-amino-acid insert. Results of transactivation comparisons between 'insert' and 'non-insert' forms are likely to be affected by different experimental procedures (Greenwood *et al.*, 2003; Lethimonier *et al.*, 2002). Differences in cell type, reporter plasmid and the use of single or multiple GREs are paramount in the resulting transactivation capacity. It is thus essential to determine how the GR splice variants of different teleostean fish species perform in a standardised transactivation assay.

2.4.6 Variation in the ligand binding region of fishes and mammals

An important region in the GR gene that can influence cortisol sensitivity is the ligand binding region. It has been shown that in rat a specific L-x-x-L-L motif (amino acids 547 to 553) at the N-terminal side of the ligand binding region is essential for Hsp90 binding. This motif forms a hydrophobic patch that contributes to the stability of the tertiary structure of the ligand binding region and thereby facilitates steroid binding. Mutations of Leu^{550} or Leu^{553} to Serine dramatically reduce steroid binding and biological activity, without altering the binding of Hsp90 (Giannoukos *et al.*, 1999). Interestingly, although Leu^{553} is conserved throughout all vertebrate species, Leu^{550} is not found in any of the teleostean species. In fact all fish GR sequences described to date have a Met at this location and duplicate GR genes also do not differ at this particular location.

Although there is little information about binding affinities of fish GRs, the affinity for dexamethasone of GR1 (K_d 5.5 nM) and GR2 (K_d 3.5 nM) were shown to be similar and comparable to human GR affinity (K_d 4.6 nM) for dexamethasone (Bury *et al.*, 2003; Ducouret *et al.*, 1995; Ray *et al.*, 1999). In common carp (*Cyprinus carpio*) lymphocytes, affinity for dexamethasone was lower (K_d 16 nM) (Weyts *et al.*, 1998b). In this experiment only one or one class of carp GR was detected, although we now know from mRNA expression data that at least two different GRs, with comparable expression profiles, are present in this fish (Stolte *et al.*, 2008a). The biochemical analysis used to determine affinity however, has insufficient resolution to confirm this. It is therefore predicted that the binding affinities of the duplicate GRs will be very comparable. Affinity for cortisol of these carp lymphocyte GRs was K_d 3.8 nM (Weyts *et al.*, 1998b). This is comparable to the affinity for cortisol of Coho salmon (*Oncorhynchus kisutch*) gill GR receptors (K_d 2.2 nM) and Chinook salmon (*Oncorhynchus tshawytscha*) brain GR receptors (K_d 4.5 nM) (Knoebl *et al.*, 1996; Maule and Schreck, 1990).

While the L-x-x-L-L motif in guinea pig (*Cavia porcellus*) is exactly the same as in rat, it has been shown to exhibit resistance to glucocorticoids that results from lower sensitivity for the ligand (Kraft *et al.*, 1979). Using a domain-swap approach the guinea pig GR ligand

binding region was inserted in the human GR gene, replacing the human ligand binding region. The chimeric GR gene product showed a decrease in hormone sensitivity of approximately 40-fold (Keightley and Fuller, 1994). In further studies, the critical regions and/or residues of the ligand binding region were identified and targeted for site-directed mutagenesis (Keightley *et al.*, 1998). A similar approach is now used in rainbow trout research (Sturm and Bury, 2005). In this case domain swap mutants were produced, not between GR genes of different species, but between the two different rainbow trout GR genes; rtGR1 and rtGR2. It was shown that the N-terminal region of rtGR1 had a more potent transactivation function than rtGR2, whereas the opposite was observed for the ligand binding region. By combinations of rtGR1 and rtGR2 domains therefore, hypersensitive and hyposensitive recombinant receptors could be obtained (Sturm and Bury, 2005).

In this way both the guinea pig and the rainbow trout provide naturally 'altered', but functional GRs (Keightley and Fuller, 1994). Domain swaps and site directed mutagenesis provide the opportunity to investigate the molecular basis of cortisol resistance. GR domain swap research within one species however, can only be performed on teleostean fishes.

2.5 Perspectives

To fully comprehend the functional implications of the different teleostean GRs we now search for (immune) cells that differentially express GRs. *In situ* hybridisation is probably the most powerful tool. To objectively compare the mammalian and the teleostean GRs they have to be tested in the same *in vitro* system. This will yield only approximations of fish receptor function as the protein is expressed in a mammalian cell with its mammalian responsive machinery. An important advantage of studies with fish is that we can study receptor function at different biologically relevant ambient temperatures as fishes are poikilotherms. This is especially interesting as it is known from studies with mammals that GR function is critically dependent on heat shock proteins/stress proteins, the expression of which is temperature dependent. However, to perform transactivation studies with a teleostean GR at different temperatures, one would need a cell type that functions properly at low temperature, preferably a fish cell type. The major drawback is that as yet no fish cell types/lines comparable to their mammalian counterparts and lacking endogenous corticoid receptors have been described.

2.6 Summary and conclusion

Teleostean fishes have duplicate GR proteins, where other vertebrates only have one. This likely results from the fish specific genome duplication event. One of these two GR genes has two different transcripts that are generated by alternative splicing. The most abundant of these two splice variant transcripts has a nine amino acid insert in its DNA binding region. This insertion is the result of transcription of an extra exon, which is located in the intron between exons 3 and 4, a feature that is unique for the GRs of teleostean fishes. Most interestingly, the three different GR forms in fish are differentially expressed *in vivo*, show different transactivational capacities, but show only slightly different affinity for their ligand. As both genes and different splice variants are transcriptionally active it is suggested that they both play an important and probably different role in the fishes' physiology.

The splice variation found in the DNA binding region of the GR gene of teleostean fishes, occurring at exactly the same location as the DNA binding region splice variation in mammals but resulting from a different mechanism, is associated with different affinity for DNA binding and different transactivation capacity. Moreover, fish with two different GR genes exhibit intraspecies genetic variation, that gives the opportunity to investigate which region of the GR gene and which residues within these regions are involved in ligand affinity and resulting transactivation acitivity. Because transactivation capacity following cortisol binding in different GR gene products does not necessarily correlate with transactivation capacity after dexamethasone binding, it is of paramount importance to check biological activity of both these ligands when a novel GR sequence is discovered. The multiple corticoid receptors in fish should provide a stimulating model to test the hypothesis of ligand exploitation (Thornton, 2001). In fish, a major function is ascribed to cortisol but also significant levels of 11-deoxycortisol and 11-deoxycorticosterone occur (Sturm *et al.*, 2005) and these ligands bind to GRs (and MRs). The notion of a far more complex signaling by related steroids and a subtle repertoire of adjusted/adjusting receptors may eventually greatly improve our understanding of fish physiology. The same notion may give rise to a better understanding of variation in glucocorticoid sensitivity in man.

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'What is it that decides when an observation is actually made and who is able to make one?'

Robert Gilmore, Alice in Quantumland

3

Corticosteroid receptors involved in stress regulation in common carp, *Cyprinus carpio*

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Abstract

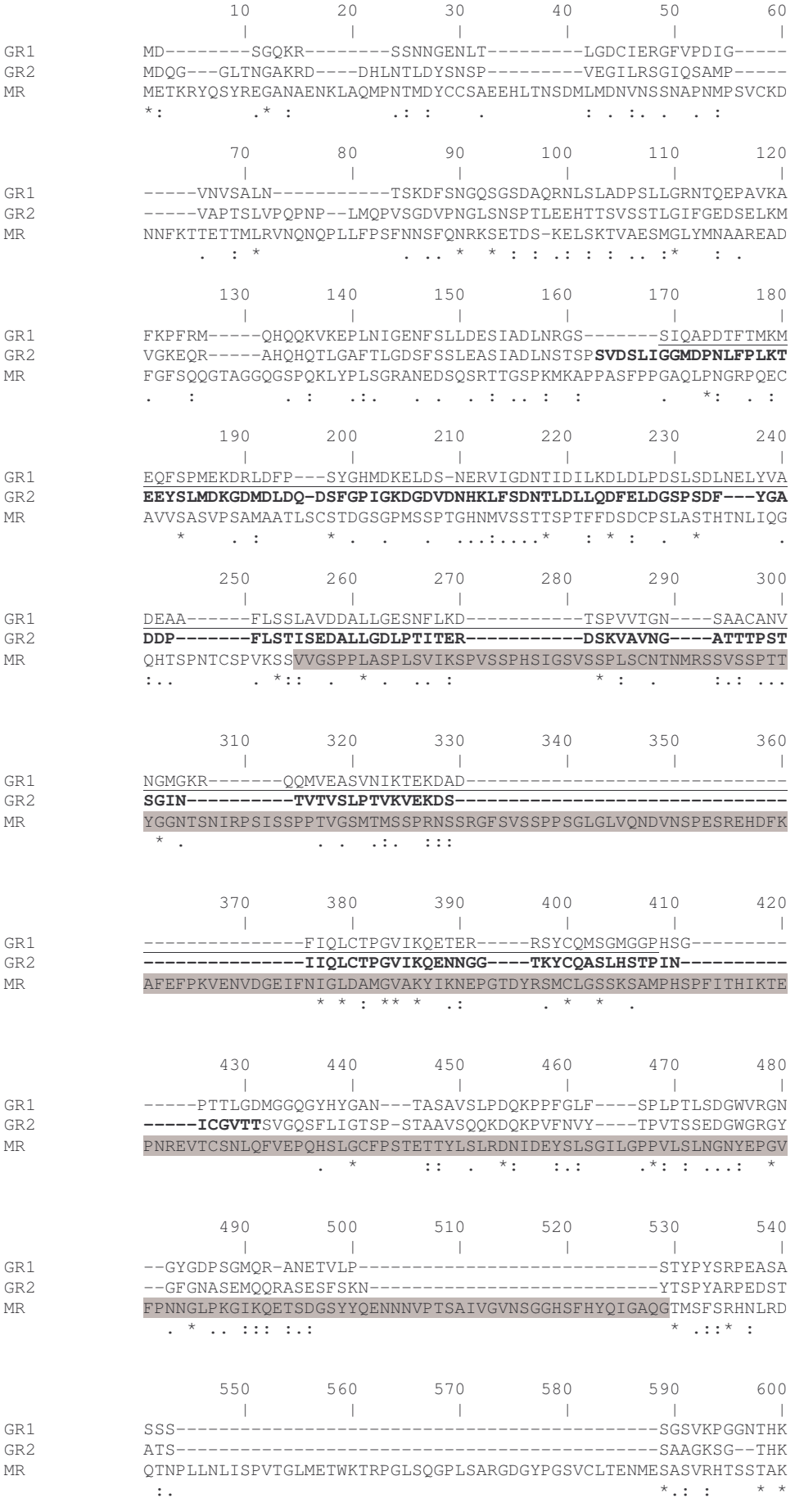
In higher vertebrates, mineralo- (aldosterone) and glucocorticoids (cortisol/corticosterone) exert their multiple actions *via* specific transcription factors, glucocorticoid (GR) and mineralocorticoid (MR) receptors. Teleostean fishes lack aldosterone and mineral regulatory processes seem under dominant control by cortisol. Despite the absence of the classical mineralocorticoid aldosterone, teleostean fishes do have a mineralocorticoid receptor (MR) with cortisol and possibly 11-deoxycorticosterone (as alternative for aldosterone) as predominant ligands. We studied corticoid receptors in common carp (*Cyprinus carpio* L.). Through homology cloning and bio-informatic analysis we found duplicated GR genes and a single MR gene. The GR genes likely result from a major genomic duplication event in the teleostean lineage; we propose that the gene for a second MR was lost. Transactivation studies show that the carp GRs and MR have comparable affinity for cortisol; the MR has significantly higher sensitivity to 11-deoxycorticosterone, and this favors a role for DOC as MR ligand in fish physiology. Messenger RNA of the GRs and the MR is expressed in forebrain (in pallial areas homologous to mammalian hippocampus), in CRH-cells in the nucleus pre-opticus (NPO) and in the pituitary pars distalis ACTH-cells, three key neural/endocrine components of the stress axis. After exposure to prolonged and strong (not to mild acute) stressors, mRNA levels of both GRs and MR become downregulated in the brain, but not in the NPO CRH-cells or pituitary ACTH-cells. Our data predict a function in stress physiology for all CRs and suggest telencephalon as a first line cortisol target in stress.

3.1 Introduction

The adrenal cortex of mammals produces cortisol (or corticosterone) as glucocorticoid and aldosterone as mineralocorticoid. The function of these steroids is ultimately specified by the transcription factors (glucocorticoid and mineralocorticoid receptors, GRs and MRs, respectively) that mediate their actions in the diverse targets and that define which genes will be activated or repressed. Interrenal cells of teleostean fishes produce cortisol as the major steroid; in addition deoxycorticosterone (DOC, a possible side-product of progesterone conversion by 21-hydroxylase activity in cortisol pathway) is found in fish plasma (Sturm *et al.*, 2005; Colombe *et al.*, 2000). Aldosterone is an evolutionary more recent steroid (Bridgham *et al.*, 2006), believed to be absent in teleostean fishes (Balment and Henderson, 1987).

In all vertebrates glucocorticosteroids play a key regulatory role in stress responses, growth and general metabolism, reproduction and immunity (Mommsen *et al.*, 1999), and in terrestrial vertebrates a specific subtask in mineral regulation is given to aldosterone. In fish, cortisol is intimately involved in the regulation of the water and mineral balance (Gilmour, 2005). At least two notions come to mind at the basis of a fundamentally different corticoid endocrinology in fishes: i) cortisol, the main corticosteroid exerts receptor-defined gluco- or mineralocorticoid actions and thus regulation of water and mineral balance in fishes is not necessarily controlled by a mineralocorticoid; ii) the poorly studied DOC could, *via* a MR, act as a mineralocorticoid in fishes.

The corticoid receptors are promiscuous for ligands; cortisol is bound both by the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The evolution of multiple corticosteroid receptors and their signaling pathways in vertebrates was extensively reviewed (Baker *et al.*, 2007; Bridgham *et al.*, 2006; Bury and Sturm, 2007; Prunet *et al.*, 2006). An ancestral corticosteroid receptor (AncCR) is assumed to have been an effective receptor for cortisol; the AncCR may further have transmitted a DOC signal in the ancestors of fishes. Duplication of the AncCR-gene led to separate GR and MR species over 450 million years ago; it is assumed that the MR retained an ancestral phenotype and that the GR lost sensitivity for aldosterone (Bridgham *et al.*, 2006) in favor of cortisol. A second major genomic duplication event took place in teleostean fishes (not in tetrapods), and gave rise to further diversification of the corticosteroid receptor family. In the extant teleostean fishes studied so far, two different GR genes and one MR gene are found (Bury and Sturm, 2007; Greenwood *et al.*, 2003). The interesting picture arises that in fish a single ligand (cortisol) may steer three different receptors; diversification therefore of receptors rather


10 20 30 40 50 60
GR1 MD-------SGQKR--------SSNNGENLT---------LGDCIERGFVPDIG-----
GR2 MDQG---GLTNGAKRD----DHLNTLDYSNSP---------VEGILRSGIQSAMP-----
MR METKRYQSYREGANAENKLAQMPNTMDYCCSAEEHLTNSDMLMDNVNSSNAPNMPSVCKD
70 80 90 100 110 120
GR1 -----VNVSALN-----------TSKDFSNGQSGSDAQRNLSLADPSLLGRNTQEPAVKA
GR2 -----VAPTSLVPQPNP--LMQPVSGDVPNGLSNSPTLEEHTTSVSSTLGIFGEDSELKM
MR NNFKTTETTMLRVNQNQPLLFPSFNNSFQNRKSETDS-KELSKTVAESMGLYMNAAREAD
130 140 150 160 170 180
GR1 FKPFRM-----QHQQKVKEPLNIGENFSLLDESIADLNRGS-------SIQAPDTFTMKM
GR2 VGKEQR-----AHQHQTLGAFTLGDSFSSLEASIADLNSTSPSVDSLIGGMDPNLFPLKT
MR FGFSQQGTAGGQGSPQKLYPLSGRANEDSQSRTTGSPKMKAPPASFPPGAQLPNGRPQEC
190 200 210 220 230 240
GR1 EQFSPMEKDRLDFP---SYGHMDKELDS-NERVIGDNTIDILKDLDLPDSLSDLNELYVA
GR2 EEYSLMDKGDMDLDQ-DSFGPIGKDGDVDNHKLFSDNTLDLLQDFELDGSPSDF---YGA
MR AVVSASVPSAMAATLSCSTDGSGPMSSPTGHNMVSSTTSPTFFDSDCPSLASTHTNLIQG
250 260 270 280 290 300
GR1 DEAA------FLSSLAVDDALLGESNFLKD----------TSPVVTGN----SAACANV
GR2 DDP-------FLSTISEDALLGDLPTITER----------DSKVAVNG----ATTTPST
MR QHTSPNTCSPVKSSVVGSPPLASPLSVIKSPVSSPHSIGSVSSPLSCNTNMRSSVSSPTT
310 320 330 340 350 360
GR1 NGMGKR-------QQMVEASVNIKTEKDAD-----------------------------
GR2 SGIN----------TVTVSLPTVKVEKDS-----------------------------
MR YGGNTSNIRPSISSPPTVGSMTMSSPRNSSRGFSVSSPPSGLGLVQNDVNSPESREHDFK
370 380 390 400 410 420
GR1 ---------------FIQLCTPGVIKQETER-----RSYCQMSGMGGPHSG---------
GR2 ---------------IIQLCTPGVIKQENNGG----TKYCQASLHSTPIN----------
MR AFEFPKVENVDGEIFNIGLDAMGVAKYIKNEPGTDYRSMCLGSSKSAMPHSPFITHIKTE
430 440 450 460 470 480
GR1 -----PTTLGDMGGQGYHYGAN---TASAVSLPDQKPPFGLF----SPLPTLSDGWVRGN
GR2 -----ICGVTTSVGQSFLIGTSP-STAAVSQQKDQKPVFNVY----TPVTSSEDGWGRGY
MR PNREVTCSNLQFVEPQHSLGCFPSTETTYLSLRDNIDEYSLSGILGPPVLSLNGNYEPGV
490 500 510 520 530 540
GR1 --GYGDPSGMQR-ANETVLP-------------------------STYPYSRPEASA
GR2 --GFGNASEMQQRASESFSKN-------------------------YTSPYARPEDST
MR FPNNGLPKGIKQETSDGSYYQENNNVPTSAIVGVNSGGHSFHYQIGAQGTMSFSRHNLRD
550 560 570 580 590 600
GR1 SSS---SGSVKPGGNTHK
GR2 ATS---SAAGKSG--THK
MR QTNPLLNLISPVTGLMETWKTRPGLSQGPLSARGDGYPGSVCLTENMESASVRHTSSTAK

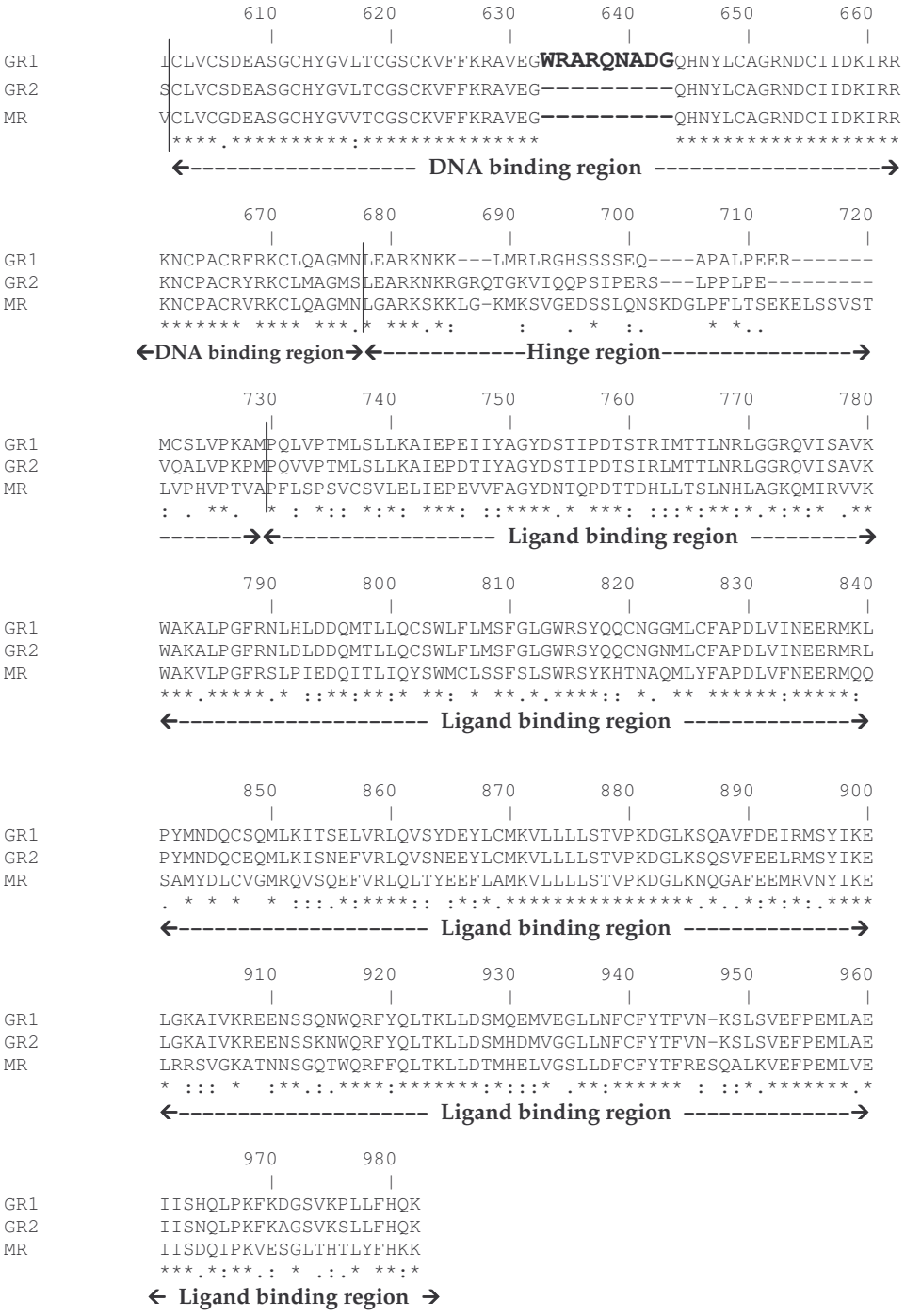


Figure 3.1: Alignment of corticosteroid receptor sequences of carp. Locations of probes are indicated; GR1 is underlined, GR2 is shown in **bold** and MR is shown in grey. Different regions of the receptors are indicated below the alignment; the transactivation region is located N-terminally from the other regions and has not been indicated. The nine amino acid insert present in the DNA binding region of GR1 is shown in a larger, bold font. Asterisks indicate conserved amino acids, double points indicate replacement by amino acids with high similarity and single points indicate replacement with amino acids of low similarity.

than of ligands.

Physiological research on the role of these fish receptors (combined) in stress and osmoregulation is as yet scarce. Although extensive research effort has been put in aspects of stress handling, smoltification, and reproduction, and the effects of increased cortisol levels on GR expression (Basu *et al.*, 2003; Maule and Schreck, 1991), only a few studies focussed on both GR and MR activity either simultaneously (Greenwood *et al.*, 2003) or within the same species (Bury *et al.*, 2003; Sturm *et al.*, 2005). A role for MR activity in fish osmoregulation seems likely (Prunet *et al.*, 2006; Sturm *et al.*, 2005; Gilmour, 2005), yet other MR-functionality, for instance in brain function, or stress axis regulation requires far more research.

The endocrine stress axis is a pivotal and phylogenetically ancient regulatory system, key in adaptation of vertebrates to their dynamic environment (Wendelaar Bonga, 1997), as well as in stress handling. The teleostean hypothalamo-pituitary-interrenal (HPI) axis is comparable to the mammalian stress axis (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997), a result of convergent evolution. Stressful sensory information is conveyed to the hypothalamic preoptic area (NPO) and results in release of corticotropin-releasing hormone (CRH). NPO CRH-cells in fish project directly to the rostral pars distalis adrenocorticotropic hormone (ACTH-) producing cells. ACTH released into the general circulation will then activate interrenal cells of the head kidney to produce and secrete cortisol. This cortisol will redistribute energy flows to deal with the stressor(s) and by doing so guarantees homeostasis (Wendelaar Bonga, 1997).

In mammals both GR and MR are involved in regulation of cortisol release and coping with stress (de Kloet *et al.*, 1998). MR activity governs a tonic hippocampal inhibitory control over the hypothalamus-pituitary-adrenal (HPA) axis; GR activity exerts a negative feedback on higher brain centres under conditions of high cortisol. (de Kloet *et al.*, 1998; Reul *et al.*, 2000). Indeed, high cortisol levels, via GR activity, inhibit CRH and ACTH release from hypothalamus and pituitary gland, respectively, to counteract stress imposed (de Kloet *et al.*, 1998). In fish such negative feedback has been described (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997), but which receptors are involved is not known nor are the signals precisely defined (*i.e.* is it cortisol, DOC or maybe both).

We therefore investigated which corticosteroid receptors were present in common carp and where these receptors were located in the stress axis and focussed on CRH- and ACTH-cells and higher brain centres (telencephalic pallial neurons). Next we determined mRNA expression of three receptors (GR1, GR2 and MR) and sensitivity for cortisol by transactivation analysis to assign possible differential functionality under basal or stress conditions

(*i.e.* when plasma cortisol is low and high). Finally we showed that corticosteroid mRNA expression in the stress axis could be manipulated by a chronic stress paradigm suggesting involvement of all three receptors in stress axis regulation.

3.2 Experimental procedures

3.2.1 Animals

Common carp (*Cyprinus carpio* L.) were kept at 23 °C in recirculating UV-treated tap water at 'De Haar Vissen' in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7 % of their estimated body weight. The cross 'R3×R8' is offspring of Hungarian (R8) and Polish (R3) strains (Irnazarow, 1995). Experimental repeats were performed with fish from different batches of eggs. All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

3.2.2 Identification of GR1, GR2 and MR genes

We screened the Ensembl zebrafish genome database with sequences of mammalian GR and MR genes, using the BLAST (basic local alignment search tool) algorithm. This screen revealed one GR gene and one MR gene. These genes were incorporated in separate multiple sequence alignments, using CLUSTALW (Chenna *et al.*, 2003); for both the GR and MR, genes of several species were used. Primers were designed in regions of high amino acid identity. We obtained partial cDNA sequences from a λ ZAP cDNA library of carp brain. By RACE (rapid amplification of cDNA ends; Invitrogen, Carlsbad, CA, USA) the corresponding full length sequences were obtained. PCR was carried out as previously described (Huising *et al.*, 2004a) and sequences were determined from both strands.

Table 3.1: Primers used to generate in situ hybridisation probes.

Gene		Sequence	Amplicon length (bp)
GR1	fw	5'-AGC-ATC-CAG-GCC-CCT-GAC-AC-3'	474
	rv	5'-AGG-ACC-ACC-CAT-CCC-TGA-CAT-CTG-3'	
GR2	fw	5'-CCT-TCA-GTG-GAC-TCC-CTG-ATT-G-3'	509
	rv	5'-GAA-GTG-GTG-ACG-CCG-CAG-ATG-TTA-A-3'	
MR	fw	5'-CAY-YGT-GGG-GTC-ACC-TCC-AC-3'	827
	rv	5'-TCC-CTT-GCG-CTC-CAA-TCT-GG-3'	

3.2.3 Probe synthesis

Digoxygenine (DIG) labelled carp GR1, GR2 and MR probes (table 3.1) were synthesised from a PGEMTeasy (Promega, Madison, WI, USA) vector with the respective inserts by use of a DIG RNA labelling kit according to the manufacturer's instructions (Roche, Applied Science, Almere, The Netherlands). The GR1 vector contained a 474 base pairs insert, the GR2 vector a 509 base pairs insert and the MR vector an 827 base pairs insert (primers shown in table 3.1). Proper probe labelling was confirmed by Northern blotting. Probes were constructed in the AB domain, the least conserved region, to avoid cross-reactivity between probes (figure 3.1).

3.2.4 Tissue and section preparation

Nine months old carp (150–200 g) were anaesthetised with 0.2 g/l tricaine methane sulfonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l $NaHCO_3$ (Merck, Darmstadt, Germany). Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe fitted with a 21 gauge needle. Next, fish were killed by spinal transsection and organs and tissues for RNA extraction were carefully removed, snap frozen in solid CO_2 or liquid N_2 and stored at -80 °C. Organs for *in situ* hybridisation were removed and fixed overnight in 4 % freshly prepared paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Next, tissues were transferred to 1.5 % agarose in 15 % sucrose in PBS, snap frozen in liquid N_2 and stored at -80 °C. Serial 7 µm cryostat sections were made (Frigocut 2800, Reichert-Jung) and mounted on poly-L-lysine-coated microscope slides (BDH Laboratory Supplies, Poole, UK).

3.2.5 *In situ* hybridisation

In situ hybridisation included a proteinase K (5 µg/ml) (Promega, Madison, WI, USA) treatment for 10 min at 37 °C to improve probe accessibility and a 10 min exposure to 0.25 % acetic acid anhydride in 0.1 M triethanolamine (TEA) to stop RNAse activity as described before (Engelsma *et al.*, 2001). Probes (sense 0.5 ng/µl and antisense 1.5 ng/µl) were dissolved in Hyb^+ buffer with 2 % blocking reagent (Roche Applied Science, Almere, The Netherlands) and denatured for 5 min at 80 °C, added to every glass slide, covered with parafilm and incubated overnight at 55 °C. Hyb^+ buffer was replaced with 4× SSCT (4× SCC with 0.01 % Tween 20) buffer and sections were rinsed (2× 15 min) in 4× SSCT buffer at 60 °C. An RNAse treatment with 10 µg/ml RNAseA (Qiagen, Venlo, The Netherlands) for 30 min at 37 °C removed unbound RNA fragments. Next, slides were washed (2× with

2× SSCT, for 5 min) at 60 °C, once with 1× SSCT for 10 min at 60 °C, once with 0.5× SSCT for 10 min at 60 °C and finally once with 0.1× SSCT for 30 min during which the samples were allowed to cool to room temperature. Washing and colour reaction were performed as described before.

3.2.6 Immunohistochemistry

Tissue on glass slides was first fixed in 4 % PFA in PBS for 15 min. Slides were washed once with PBST for 5 min and once with aquadest for 5 min. Subsequently they were incubated 10 min with methanol plus 0.3 % H_2O_2, after which the slides were rinsed twice for 10 min in PBST. Subsequently non-specific antigenic sites were blocked with 10 % normal goat serum (NGS) in PBS for 30 min. The slides were incubated overnight with polyclonal antiserum against Growth Hormone (1:4000) or ACTH (1:2000) in PBS with 10 % NGS. The next day the slides were rinsed twice for 10 min in PBST and incubated for 1 hr with goat anti-rabbit secondary antibody at a dilution of 1:200.

3.2.7 Imaging

Pictures were taken with Zeiss Axiovert tv 135 microscope with a 5.0 Q-imaging colour camera and Leitz orthoplan cool snap colour camera (Roper Scientific). Pictures were edited (cropped and background colour compensation) using Adobe Photoshop.

3.2.8 Stress experiments

Restraint stress Prolonged restraint (24 hr) was given by netting the fish and suspending the nets with the fish in the tanks (Huising *et al.*, 2004a). After 24 hr, the experimental group was transferred all at once to a tank with 0.2 g/l TMS, resulting in rapid (< 1 min) and deep anaesthesia prior to blood sampling and killing. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anaesthesia, immediately before sampling of the experimental group.

Cold water stress Fish were netted and transferred from a tank with 23 °C water to an identical tank with 10 °C water, and left there for 15 min, after which they were returned to their original tank. This transfer was repeated three times a day for three days. At day 4, the fish were transferred once more and sampled 30 min after return to their original (warm) tank. For sampling, fish of a tank were all at once transferred to a tank with 0.2 g/l TMS, resulting in rapid anaesthesia. Sham-treated fish were housed in identical tanks and transferred as mentioned above, but to tanks with 23 °C water. Control fish were housed in

identical tanks and left undisturbed. The sham and control fish were sampled just before sampling of experimental fish.

3.2.9 Physiological parameters and plasma hormone determination

Freshly collected, heparinised blood was centrifuged for 10 min at 2000 *g* at 4 °C, after which plasma was transferred to a new tube and stored at -20 °C. Cortisol was measured by RIA (Arends *et al.*, 1998), with a commercial antiserum (Bioclinical Services Ltd, Cardiff, UK). All constituents were in phosphate-EDTA buffer (0.05 M Na_2HPO_4, 0.01 M Na_2EDTA, 0.003 M NaN_3, pH 7.4). Ten-microliter samples or standards in RIA buffer (phosphated-EDTA buffer containing 0.1 % 8-anilia-1-naphthalene sulphonic acid and 0.1 % w/v bovine γ-globulin) were incubated with 100 µl antiserum (in RIA buffer containing 0.2 % normal rabbit serum) for 4 hr. Samples were incubated overnight with 100 µl iodinated cortisol approximately 1700 cpm/tube; ^{125}I-labelled cortisol, Amersham; Uppsala, Sweden) and 100 µl goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol in the assay were separated by the addition of 1 ml ice-cold precipitation buffer (phosphate-EDTA buffer containing 2 % w/v bovine serum albumin and 5 % w/v polyethylene glycol). Tubes were centrifuged at 4 °C (20 min at 2000 *g*), the supernatant aspirated and counted in a gamma counter (1272 clinigamma, LKB, Turku, Finland). Plasma glucose and Na^+ levels were measured with a Stat Profile® pHOx® Plus L Analyser (Nova Biochemical, Waltham, USA).

3.2.10 RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen, Carlsbad, CA, USA), as suggested by the manufacturer. Total RNA was precipitated in isopropanol, washed with 75 % ethanol and dissolved in nuclease-free water. RNA of separated pituitary glands (pars distalis and pars intermedia separate) was isolated as described by the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) strictly according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5 % agarose gel before proceeding with cDNA synthesis.

3.2.11 DNAse treatment and first strand cDNA synthesis

For each sample a '–RT' (non reverse transcriptase) control was included. One microliter of 10× Dnase-I reaction buffer and 1 µl Dnase-I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65 °C for 10 min. To each sample, 300 ng random

Table 3.2: Primers used for gene expression studies.

Gene		Sequence	Amplicon length (bp)	GenBank Acc. No.
GR1	fw	5'AGA-CTG-AGA-GGC-GGA-GCT-ACT-G-3'	113	AJ879149
	rv	5'GGC-GGT-GTT-GGC-TCC-AT-3'		
GR2	fw	5'GGA-GAA-CAA-CGG-TGG-GAC-TAA-AT-3'	110	AM183668
	rv	5'GGC-TGG-TCC-CGA-TTA-GGA-A-3'		
MR	fw	5'TTC-CCT-GCA-GAA-CTC-AAA-GGA-3'	117	AJ783704
	rv	5'ACG-GAC-GGT-GAC-AGA-AAC-G-3'		
PRL	fw	5'CAT-CAA-TGG-TGT-CGG-TCT-GA-3'	130	X52881
	rv	5'TGA-AGA-GAG-GAA-GTG-TGG-CA-3'		
β-actin	fw	5'GCT-ATG-TGG-CTC-TTG-ACT-TCG-A-3'	89	M24113
	rv	5'CCG-TCA-GGC-AGC-TCA-TAG-CT-3'		
40S	fw	5'CCG-TGG-GTG-ACA-TCG-TTA-CA-3'	69	AB012087
	rv	5'TCA-GGA-CAT-TGA-ACC-TCA-CTG-TCT-3'		

hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5× First Strand buffer, 2 µl 0.1 M dithiothreitol (DTT) and 40 Units RNAse Out (Invitrogen 10777-019) were added and the mix was incubated for 10 min at room temperature and for and additional 2 min at 37 °C. To each sample (not to the '–RT' controls) 200 U Superscript-II RNase H^- Reverse Transcriptase (RT; Invitrogen, 18064-014) was added and reactions were incubated for 50 min at 37 °C. Demineralised water was added to a final volume of 100 µl and stored at -20 °C until further use.

3.2.12 Real-time quantitative PCR

PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and *PRIMER3* software (Rozen and Skaletsky, 2000) was used to design primers for use in real-time quantitative PCR (RQ-PCR) (table 3.2). For RQ-PCR 5 µl cDNA and forward and reverse primers (300 nM each) were added to 12.5 µl Quantitect Sybr Green PCR Master Mix (Qiagen, Venlo, The Netherlands) and filled up with demineralised water to a final volume of 25 µl. RQ-PCR (15 min 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C followed by 1 min at 60 °C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed by use of the comparative quantitation of the Rotor-gene Analysis Software V5.0. Basal gene expression in organs and tissues of

adult carp was determined as a ratio of target gene vs. reference gene and was calculated according to the following equation.

$$\text{ratio} = \frac{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}}}{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}}} \tag{3.1}$$

where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value. Expression following 24 hr of restraint or cold water transfer was determined relative to the expression of non-restraint control fish according to the following equation (Pfaffl, 2001).

$$\text{ratio} = \frac{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}(\text{control} - \text{sample})}}{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}(\text{control} - \text{sample})}} \tag{3.2}$$

Dual internal reference genes (40S ribosomal protein and β-actin) were incorporated in all RQ-PCR experiments; results were similar following standardization to either gene. '–RT'-controls were included in all experiments and no amplification above background levels was observed. Non-template controls were included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

3.2.13 Transactivation assay

Clones encoding full-length open reading frame of common carp GR1 and GR2 were excised from pGEM-Teasy vector by EcoR1 and BamH1 and ligated into pcDNA3 expression vector cut with the same enzymes. Orientation and quality of the insert was confirmed by sequencing. COS-7 cells (derived from African green monkey kidney) were cultured as described previously (Sturm *et al.*, 2005). Cells were transiently transfected using a calcium precipitation method (Sambrook and Russell, 2001), with cells growing in log phase at 30–50 % confluence. Cells were co-transfected with the following plasmids: expression vector with the appropriate hormone receptor cDNA, (1 µg / 24 well plate), reporter plasmid pFC31Luc, which contains the mouse mammary tumour virus promoter upstream of the luciferase gene (MMTV-LUC) (10 µg / 24 well plate), and pSVβ (Clontech, Palo Alto, CA, USA), a second reporter plasmid under control of the SV40 promoter and serving as a control for transfection efficiency (2 µg / 24 well plate) and finally pBluescript (Clontech, Palo Alto, CA, USA) (7 µg / 24 well plate), an irrelevant plasmid to increase transfection. Sixteen hours after transfection, medium was renewed and cortisol added from 1000-fold

concentrated stock solution in ethanol. After 36 hr incubation, cells were harvested using reporter lysis buffer (Promega, Madison, WI, USA) following the manufacturer's instructions; luciferase and β-galactosidase activities were determined as described previously (Bury *et al.*, 2003). In addition to solvent controls (receiving only ethanol carrier instead of hormone) cells were transfected with an empty expression vector to control for luciferase activity in absence of hormone receptor DNA. Experiments were repeated three times independently, with triplicate cell cultures per treatment. Luciferase activity was corrected for 'well-specific' transfection efficiency (determined by β-galactosidase activity) and then expressed as percentage luciferase activity observed in cells treated with 10^{-7} M cortisol.

Half maximum activation concentration of ligand (EC_{50}) in the transactivation assay was assessed by fitting the data to a single ligand binding model using Sigma plot® software. Only converging data were included in data sets presented. Ligands were tested in the range of 10 pM to 1 μM. Data were normalised to maximum (100 %) response and corrected for blanks prior to kinetic analysis.

3.2.14 Bioinformatics

Sequences were retrieved from the Swissprot, EMBL and GenBank databases using SRS and/or BLAST (basic local alignment search tool) (Altschul *et al.*, 1997). Multiple sequence alignments were carried out using CLUSTALW (Chenna *et al.*, 2003). Calculation of pairwise amino acid identities was carried out using the SIM ALIGNMENT tool (Huang and Miller, 1991). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004). Phylogenetic tree was constructed based on the neighbour-joining method using the Poisson-correction for evolutionary distance (Nei and Kumar, 2000). Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

3.2.15 Statistics

Statistic analysis was performed with SPSS 12.0.1 software. Following ANOVA, differences between treatments were assessed by Mann-Whitney U test, and $P < 0.05$ was accepted as fiducial limit. For RQ-PCR data, tests were performed for both internal reference genes (β-actin and 40S) and statistical significance is only reported if both reference genes showed a significant effect, where * indicates $P < 0.05$ and ** indicates $P < 0.01$.

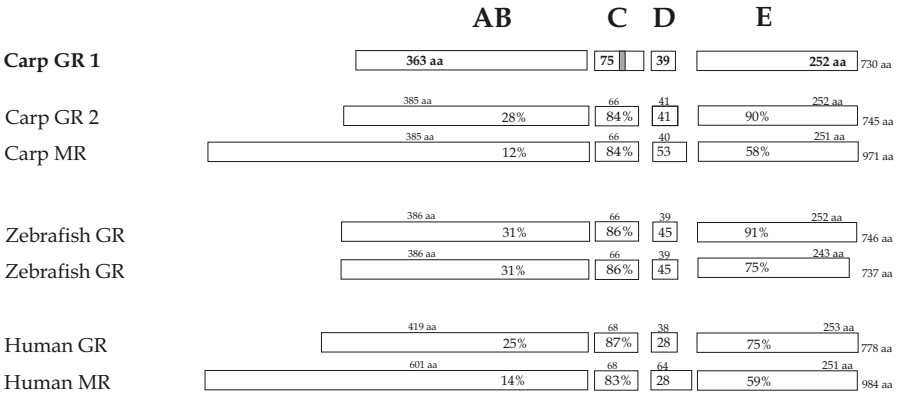


Figure 3.2: Similarities between the receptor domains (AB ∼ transactivation region, C ∼ DNA-binding region, D ∼ hinge region and E ∼ ligand binding region) of the common carp and other vertebrate corticosteroid receptors. Percentage amino acid identity of the different domains is shown in the boxes. Amino acid length of particular domains is represented by length of boxes and is also mentioned. Total length of each protein is shown at the right. Grey bar indicates 9 amino acid insert as a result of alternative splicing.

3.3 Results

3.3.1 CR characterisation

Cloning and characterization of the MR and GR genes. Full-length sequences of one MR and two different GR genes were obtained by homology cloning using a common carp brain cDNA library. The first GR gene (GR1; Acc. No. AJ 879149) comprises 2190 nucleotides, and encodes a protein of 730 amino acids. The second gene (GR2; Acc. No. AM183668) contains an open reading frame of 2235 nucleotides, and encodes a protein of 745 amino acids (figure 3.1). The predicted amino acid identity of these two GRs is 57 %; both gene products show moderate sequence (45–60 %) identity when compared to other teleostean fish and mammalian GR genes (figure 3.2). The MR gene (Acc. No. AJ783704) has an open reading frame of 2913 nucleotides, which codes for a 971 amino acids protein. The predicted MR amino acid sequence shows relatively high (65–90 %) sequence identity to other teleostean fish MR sequences and moderate (≈50 %) sequence identity to the African clawed frog (*Xenopus laevis*) and mammalian MR sequences.

When the protein domains of the receptors (GRs and MR) are compared among different species, 85–100% sequence identity is found for the DNA binding domain. For the ligand binding domain (LDB) 50–60 % sequence identity is found when GRs are compared to MRs between species, and 70–90 % when LDBs of either GRs or MR of individual species are compared (figure 3.2). The N-terminal domains of GRs and MRs constitute the most variable region. Alignment of carp GR- and MR-genes yields low sequence conservation

(28 % for GR1 compared to GR2 and < 15 % for MR compared to either of the GRs; figure 3.1).

Phylogenetic analysis. The neighbour-joining phylogenetic tree for corticosteroid receptor proteins (figure 3.3) resulted in a predicted cluster of GRs and MRs on separate branches together with mammalian orthologues; androgen receptors and corticosteroid receptors from jawless fish represent an outgroup. Within both the MR and the GR branch, teleostean and tetrapod proteins form separate clades. The teleostean GR clade has a subdivision as a result of duplication of the GR gene.

Expression of mRNAs for corticosteroid receptors. Expression of GR1-, GR2- and MR-genes in brain (without hypothalamus and pituitary gland), ventral hypothalamus, pituitary gland of healthy, non-stressed carp was quantitated by real time PCR is given in figure 3.4. The highest GR expression was found in brain and hypothalamus. Expression of GR1- and GR2-genes was generally comparable; in brain, however, GR1 expression was higher than GR2 expression. Receptor expression abundance in pituitary tissue was about half that seen in brain or hypothalamus. The MR gene also showed an about 50 % lower expression level in pituitary tissue compared to brain and hypothalamus (figure 3.4a).

To discriminate gene expression levels in the pars distalis (POMC-cells producing ACTH) and pars intermedia (POMC-cells producing melanocyte stimulating hormone (MSH)) we dissected pituitary glands and confirmed tissue separation by assay of prolactin mRNA expression, a marker for the rostral pars distalis (RPD), (insert figure 3.4b). GR1 and GR2 mRNA expression was significantly ($P < 0.01$) higher in pituitary pars distalis compared to pars intermedia. No such difference was observed for MR mRNA (figure 3.4b).

CR localisation. The localisation of mRNA expression was further studied by *in situ* hybridisation. In the telencephalon, mRNA expression of all three receptors were detected, predominantly in the outer pallial layers. In other brain areas GR1 and GR2 show the same distribution pattern, although relative expression levels were different, whereas MR mRNA showed a less wide distribution. In transverse sections of the hypothalamus mRNA expression of all three receptors was observed in the magnocellular part of the NPO (as assessed by comparison to paramedian sagittal slides (Huising *et al.*, 2004a)); expression was less pronounced in the parvocellular part (figure 3.5). In the pituitary pars intermedia GR mRNA expression was low. In the pars distalis, strongest GR expression was found in the proximal pars distalis, in growth hormone (GH) producing cells. In the rostral pars distalis ACTH cells express both corticosteroid receptors GR1 and GR2 and to a far higher degree than the prolactin cells (figure 3.6).

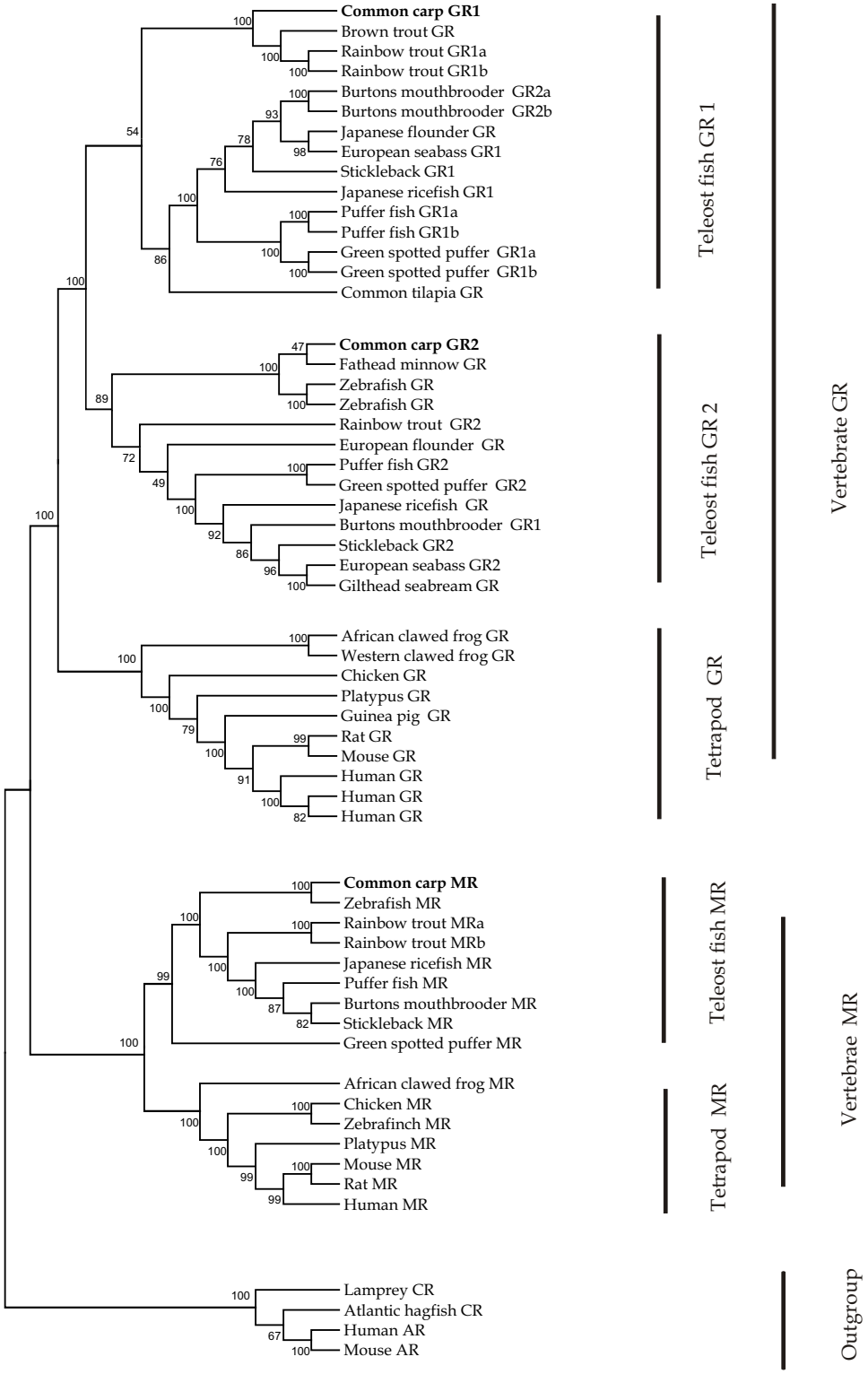
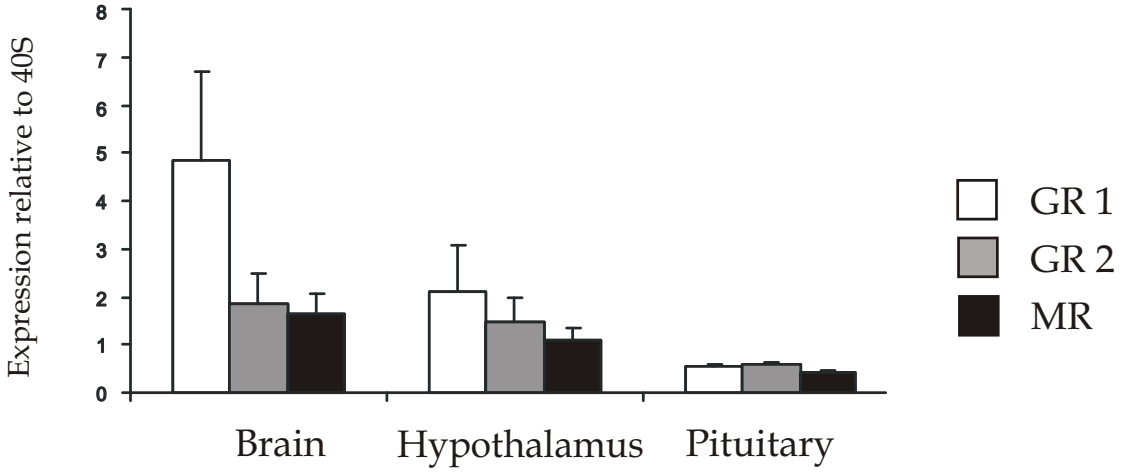
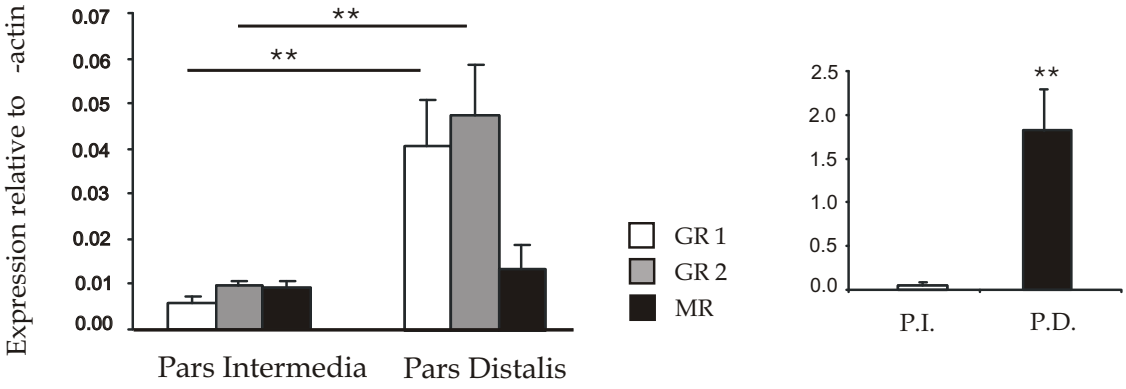

Common carp GR1
Brown trout GR
Rainbow trout GR1a
Rainbow trout GR1b
Burtons mouthbrooder GR2a
Burtons mouthbrooder GR2b
Japanese flounder GR
European seabass GR1
Stickleback GR1
Japanese ricefish GR1
Puffer fish GR1a
Puffer fish GR1b
Green spotted puffer GR1a
Green spotted puffer GR1b
Common tilapia GR
Common carp GR2
Fathead minnow GR
Zebrafish GR
Zebrafish GR
Rainbow trout GR2
European flounder GR
Puffer fish GR2
Green spotted puffer GR2
Japanese ricefish GR
Burtons mouthbrooder GR1
Stickleback GR2
European seabass GR2
Gilthead seabream GR
African clawed frog GR
Western clawed frog GR
Chicken GR
Platypus GR
Guinea pig GR
Rat GR
Mouse GR
Human GR
Human GR
Human GR
Common carp MR
Zebrafish MR
Rainbow trout MRa
Rainbow trout MRb
Japanese ricefish MR
Puffer fish MR
Burtons mouthbrooder MR
Stickleback MR
Green spotted puffer MR
African clawed frog MR
Chicken MR
Zebrafinch MR
Platypus MR
Mouse MR
Rat MR
Human MR
Lamprey CR
Atlantic hagfish CR
Human AR
Mouse AR
Teleost fish GR 1
Teleost fish GR 2
Tetrapod GR
Teleost fish MR
Tetrapod MR
Vertebrate GR
Vertebrae MR
Outgroup

Figure 3.3: Phylogenetic tree, comparing the amino acid sequences of the vertebrate corticosteroid receptors. This tree was generated with MEGA version 3.1 software using the neighbour-joining method. Reliability of this tree was assessed by bootstrapping using 1000 bootstrap replications; values in percentage are indicated at branch nodes. Atlantic hagfish and sea lamprey corticosteroid receptors (CR) and human and rat androgen receptors (AR) are used as outgroup. Common carp (*Cyprinus carpio*) GR1; AJ879149, GR2; AM183668, Rainbow trout (*Oncorhynchus mykiss*) GR1; P49843, GR2; AY4953720, Burton's mouthbrooder (*Haplochromis burtoni*) GR1; AF263738, GR2a; AF263739, GR2b; AF263740, Zebrafish (*Danio rerio*) GR2; EF436284, GR2β; EF436285, Japanese flounder (*Paralichthys olivaceus*) GR; (O73673), European sea bass (*Dicentrarchus labrax*) GR1; AY549305, GR2; AY619996, Brown trout (*Salmo trutta*) GR; AY863149, Fathead minnow (*Pimephales promelas*) GR; AY533141, Puffer fish (Fugu) (*Takifugu rubripes*) GR1; GENSCAN00000003615 (scaffold 1264) & GENSCAN00000029451 (scaffold 4328), GR2; SINFRUG00000143550 (scaffold 59), Green spotted puffer (Tetraodon) (*Tetraodon nigroviridis*) GR1; GIDT00024792001 (Chr. 7), GR2; GSTENG00017027001 (Chr. 1), Stickleback *(Gasterosteus aculeatus)* GR1; ENSGACP00000027400, GR2; ENSGACP00000024074, Japanese ricefish (Medaka) (*Oryzias latipes)* GR1; ENSORLP00000001939, GR2; ENSORLP00000007570, Mozambique or common tilapia (*Oreochromis mossambicus*) GR; BAA23662, African clawed frog (*Xenopus laevis*) GR; P49844, Western clawed frog (*Xenopus tropicalis*) GR; CR848477, Chicken (*Gallus gallus*) GR; ENSGALP00000011948 (Q8JHA4 partial), Platypus *(Ornithorhynchus anatinus)* GR; ENSOANP00000009152, Pig (*Sus scrofa*) GR; AY779185, Cow (*Bos taurus*) GR; AY238475, Guinea pig (*Cavia porcellus*) GR; P49115, Mouse (*Mus musculus*) GR; P06537, Rat (*Rattus norvegicus*) GR; NP_036708, Human (*Homo sapiens*) GR-α; P04150, Human GR-β; NP_001018661, Human GR-γ; NP_001019265, Carp (*Cyprinus carpio*) MR; AJ783704, Zebrafish (*Danio rerio*) MR; ENSDARP00000053817, Rainbow trout (*Oncorhynchus mykiss*) MRa; Y495584, MRb; AY495585, Burton's mouthbrooder (*Haplochromis burtoni*) MR; Q8JJ89, Puffer fish (Fugu) (*Takifugu rubripes*) MR; NEWSINFRUP00000129848, Green spotted puffer (Tetraodon) (*Tetraodon nigroviridis*) MR; GSTENT00032894001, Stickleback (*Gasterosteus aculeatus)* MR; ENSGACP00000022713, Japanese ricefish (Medaka) (*Oryzias latipes)* MR; ENSORLT00000009439, Chicken (*Gallus gallus*) MR;ENSGALP00000016283 (Q8QHI2, partial), Platypus (*Ornithorhynchus anatinus*) MR; ENSOANT00000008378, African clawed frog (*Xenopus laevis*) MR; BC081082, Mouse MR; XP_356093, Rainbow trout MR; AY495584, Human MR; M16801, Sea lamprey (*Petromyzon marinus*) CR; AY028457, Atlantic hagfish (*Myxine glutinosa*) CR; DQ382336, Mouse (*Mus musculus*) AR; M37890, Human (*Homo sapiens*) AR; P10275. NB: Burton's mouthbrooder nomenclature is different; GR1 has no splice variants (comparable to common carp and rainbow trout GR2) and GR2 has two splice variants (comparable to rainbow trout GR1); Burton's mouthbrooder GR2b has a nine amino acid insert.

Transactivation activity. GR2 was more sensitive to the different hormones tested than GR1. For both receptors dexamethasone was the strongest agonist tested, followed by cortisol, deoxycortisol and corticosterone. Aldosterone and DOC finally, were very weak agonists. The physiologically important stress hormone cortisol was chosen as ligand to compare sensitivity between the different receptors in a transactivation assay. Carp GR1 was less sensitive than the GR2, both to cortisol (EC_{50} 7.1 ± 2.9 nM and 2.4 ± 0.4 nM for



(a) Stress axis organs



(b) Pars distalis and pars intermedia of pituitary

Figure 3.4: Constitutive corticosteroid receptor expression in stress axis of control fish (a) (n = 12). Comparison of quantitative real time PCR data was based on samples of four untreated fish and controls from 24 hr netting experiment (n = 4) and controls of the cold water transfer experiment (n = 4). Data are plotted as average of the mean of each of the 3 experiments relative to the housekeeping gene 40S; error bars indicate standard error of the means of these 3 experiments. NB: Gene expression data relative to housekeeping gene β-actin (n = 8) showed the same pattern (not shown). Constitutive corticosteroid receptor expression in different sections of the pituitary gland (n = 5) (b). Insert shows prolactin mRNA expression of five control fish in different sections of pituitary.

Table 3.3: Transactivation capacity of corticosteroid receptors. Table shows EC_{50} values of GR1, GR2 and MR for different hormones. Cortisol for GRs; average of three separate experiments with standard error of the mean; cortisol for MR, dexamethasone, aldosterone and DOC; single experiments. DOC; 11-Deoxycorticosterone. * GR2 is significantly more sensitive to cortisol than GR1 ($P < 0.05$).

	Cortisol EC_{50} (nM)	SE	Dexamethasone EC_{50} (nM)	SD	Aldosterone EC_{50} (nM)	SD	DOC EC_{50} (nM)	SD
GR1	7.1	2.9	2.4	3.8				
GR2	2.4*	0.4	0.7	1.4				
MR	4.1	2.0			0.46	4.1	0.25	3.6

GR1 and GR2, respectively) and to dexamethasone (EC_{50} 2.4 ± 3.8 nM and 0.7 ± 1.4 nM for GR1 and GR2, respectively) (table 3.3). The carp MR has intermediate sensitivity for cortisol (EC_{50} 4.1 ± 2.0 nM) when compared to the GRs. The sensitivity of the MR to aldosterone and DOC was comparable, and approximately 10-fold higher than to cortisol.

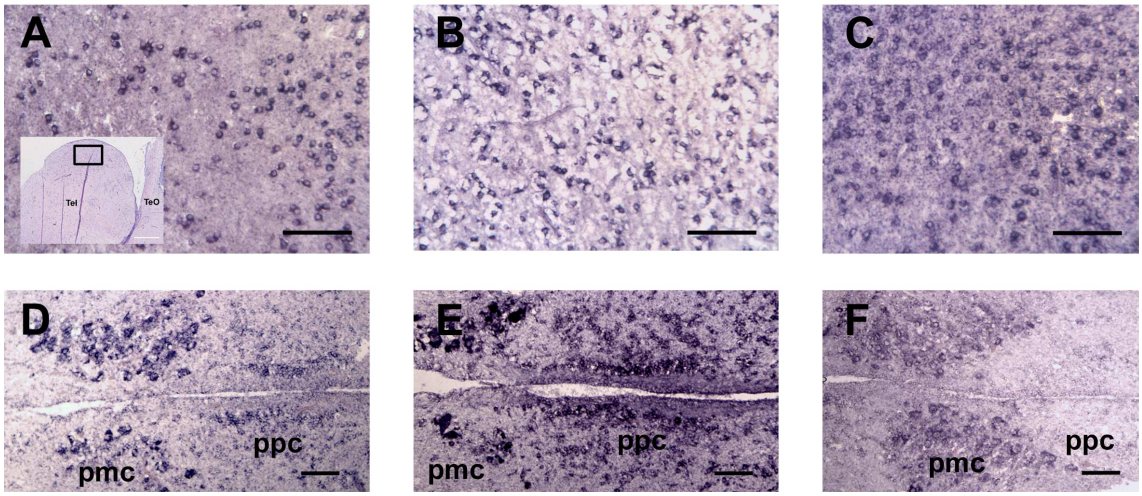


Figure 3.5: Localisation of GR1, GR2 and MR expression by ISH in cells of the telencephalon and the NPO. Panel **A**–**C**; telencephalon; (**A**); insert; overview of the telencephalon with haematoxilin and eosin staining. Anti-sense probe for GR1(**A**), GR2 (**B**) and MR (**C**). Panel **D**–**F**; NPO; nucleus pre-opticus of hypothalamus; anti-sense probe for GR1 (**D**), GR2 (**E**) and MR (**F**). Tel; telencephalon, TeO; tectum opticum, pmc; nucleus pre-opticus magnocellularis, ppc; nucleus pre-opticus parvocellularis. Scale bars indicate 500 µm (**A** insert) or 100 µm (**A**–**F**).

3.3.2 Physiology

Corticosteroid receptor expression and stress. To study corticosteroid receptor expression after stress, we used two different stress paradigms. Restraint of carp for 24 hr resulted in

significantly elevated plasma cortisol and plasma glucose values as assessed upon completion of the treatment (table 3.4a). Plasma sodium concentrations had significantly decreased in stressed animals, indicative of stress related loss of integumental permeability to water and ions (Metz *et al.*, 2003; Wendelaar Bonga, 1997). Corticosteroid receptor expression in any of the tissues tested was not affected by this restraint (figure 3.7a). When fish were repeatedly subjected to temperature drops, they showed strongly elevated plasma cortisol levels (table 3.4b) and a downregulation of mRNA expression of the GRs and the MR in brain; in hypothalamus and pituitary gland no statistically significant changes were seen (figure 3.7b).

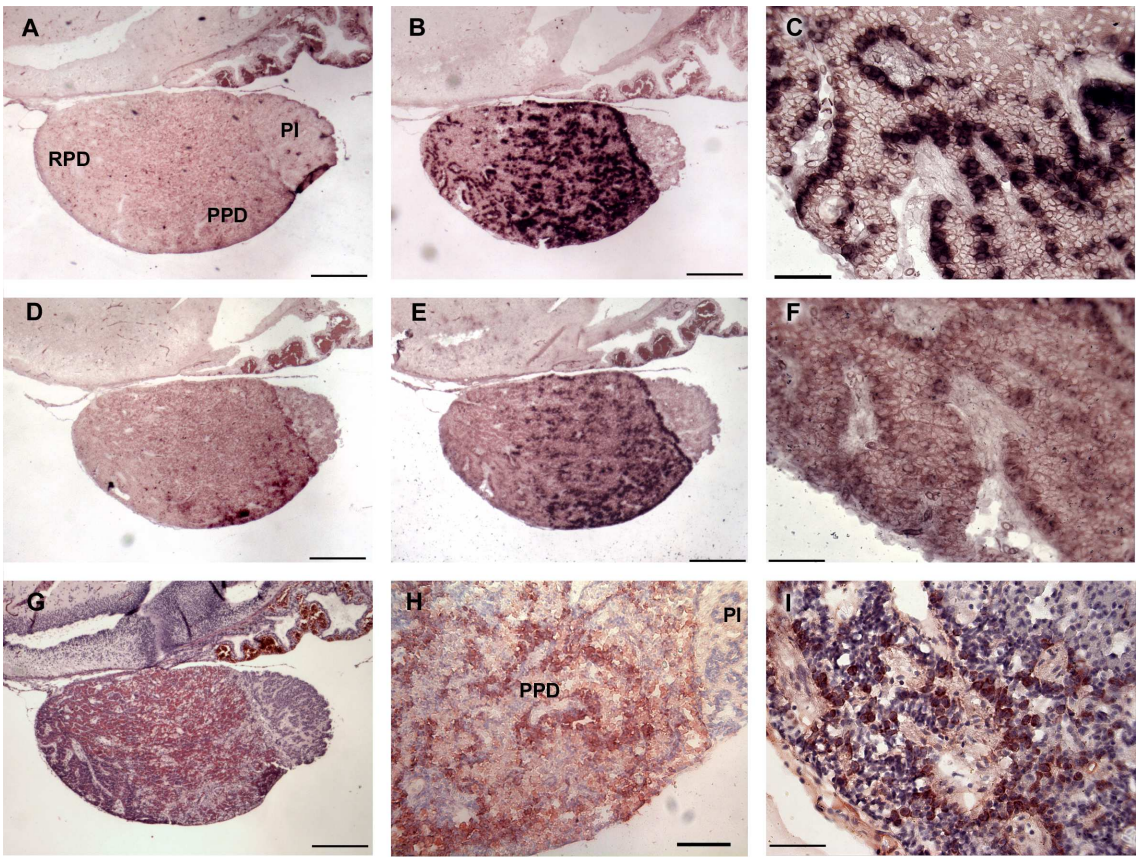


Figure 3.6: Localization of GR1 and GR2 expression in cells of the pituitary. Panel **A**; sense control for GR1, **B**; anti-sense probe for GR1, **C**; detail of rostral pars distalis (RPD) with anti-sense probe for GR1. Panel **D**; sense control for GR2, **E**; anti-sense probe for GR2, **F**; detail of RPD with antisense probe for GR2. Panel **G**; overview of pituitary stained with haematoxilin and eosin, **H**; detail of proximal pars distalis (PPD) with antibody against GH, **I**; detail of RPD with antibody against ACTH. PI; pars intermedia. Scale bars indicate 500 µm (**A, B, D, E, G**), 50 µm (**H**), 100 µm (**C, F, I**).

Table 3.4: (a) Plasma cortisol, glucose and sodium values after 24 hr restraint stress. Plasma samples of 6 control and 7 stressed fish were analysed. Data are plotted as average ± standard deviation and differences between groups were analysed by a students *t*-test. (b) Plasma cortisol, glucose and sodium values after cold transfer (23 °C to 10 °C) stress. Plasma samples of 7 control and 7 stressed fish were analysed. Data are plotted as average ± standard deviation and differences between groups were analysed by a students *t*-test. * indicates $P < 0.05$, ** represents $P < 0.01$.

(a) 24 hr restraint stress

	Control	24 hr restraint
Plasma cortisol (ng/ml)	42.6 ± 29.6	133.9 ± 33.7 *
Plasma glucose (mmol/l)	3.4 ± 0.7	8.0 ± 1.9 **
Plasma sodium (mmol/l)	126.0 ± 2.0	115.2 ± 2.4 **

(b) Cold transfer stress

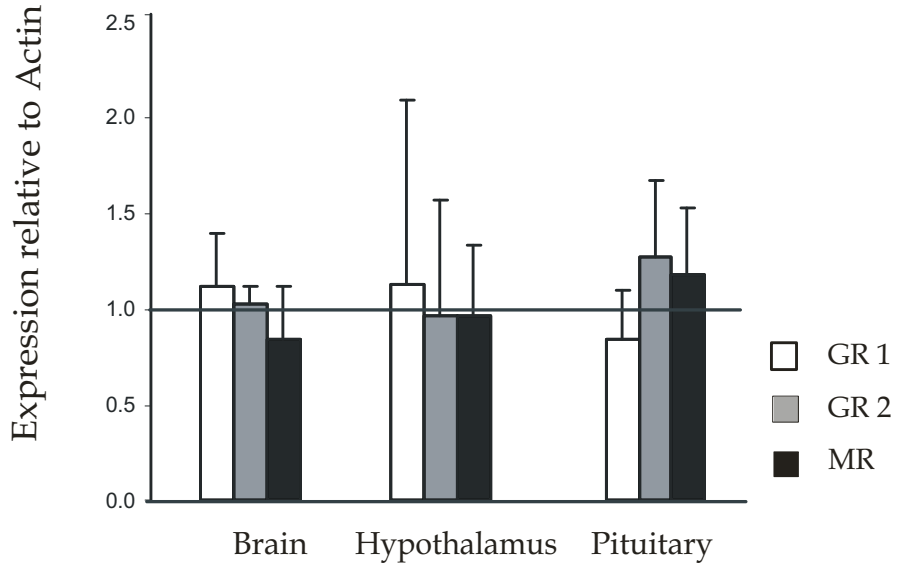
	Control	Cold transfer
Plasma cortisol (ng/ml)	3.7 ± 4.6	166.9 ± 27.1 *
Plasma glucose (mmol/l)	4.6 ± 2.5	4.3 ± 0.7
Plasma sodium (mmol/l)	130.8 ± 10.3	139.9 ± 6.1

3.4 Discussion

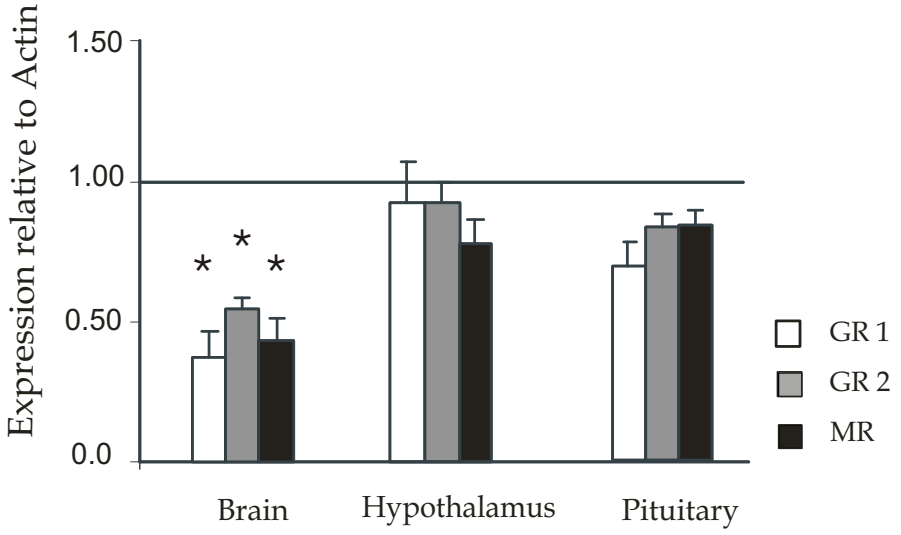
In vertebrates, corticosteroids are deeply involved in general metabolism, stress adaptation, reproduction, osmoregulation, growth and bone formation. The phenomenal pleiotropy of corticosteroids would suggest radiation of multiple isoforms and splice variants for their receptors during evolution. Indeed, research on mammals has focused on isoform and splice variant incidence of cortisol receptors (glucocorticoid receptors, GRs) and how these variants translate for instance into regulation of the stress axis. The extant teleostean fishes are representatives of the earliest true vertebrates, and exhibit a complex receptor profile. With two genes encoding functionally different GRs, this system is even more complex than that observed in mammals, which warranted investigation into the role of these different receptors in stress axis regulation.

3.4.1 CR characterisation

Receptor evolution in fishes. Different GR genes were demonstrated in distantly related teleostean species (Bury *et al.*, 2003; Greenwood *et al.*, 2003) and for that very reason not necessarily result from the tetraploidisation of common carp (Greenwood *et al.*, 2003;



(a) 24 hr restraint stress



(b) Cold transfer stress

Figure 3.7: Corticosteroid receptor expression in stress exposed fish. Gene expression of corticosteroid receptors in stress axis organs after 24 hr restraint stress (a) and cold transfer (23 °C to 10 °C) stress (b). Gene expression of 4 experimental fish is shown in comparison to 4 unstressed control fish of the respective experiment and relative to β-actin. * indicates $P < 0.05$.

Stolte *et al.*, 2006). In the green-spotted puffer two duplicates on different chromosomes are found, which makes a single gene duplication less likely (Stolte *et al.*, 2006). Moreover, all known teleostean GR1 proteins share a conserved insert of nine amino acids (WRARQNTDG, or WRARQNADG in carp) in the DNA-binding domain that is not found in other vertebrates. We rate it highly unlikely that all teleostean fishes independently duplicated a single gene and inserted every time again a nine amino acid sequence. Most

convincing for this debate is that our phylogenetic analysis yields two distinct clades of GR genes in the teleostean lineage, which argues against duplication in the tetrapod lineage. The duplication in all likelihood results from an early genome duplication 450–300 million years ago, and only after the divergence of the tetrapods from the fish lineage (Volff, 2005). If we proceed from a notion of a major genomic duplication in fishes, it follows that one MR has apparently been lost during evolution as we were not able to detect a second MR coding gene in carp or in genomic databases for zebrafish (*Danio rerio*), puffers (*Fugu* species) or rice fish (*Oryzias latipes*). Three possibilities arise after a gene/genome duplication. Non-functionalisation, which is the fate of most duplicated genes (Brunet *et al.*, 2006), neo-functionalisation; the acquirement of a new function, or sub-functionalisation; where each copy loses part of the ancestral function and both copies are required to maintain the full function (Force *et al.*, 1999). The duplicated GR genes of fish escaped a fate as non-functional pseudogene: expression levels and differential sensitivities for cortisol are more so in line with neo- or subfunctionalisation (Bury *et al.*, 2003; Greenwood *et al.*, 2003). Interestingly, zebrafish has only one GR copy that clusters with other fish GR2 sequences. However, zebrafish has acquired a splicing β-isoform of the GR (figure 3.2), that resembles the dominant negative GR-β of humans in structure, expression level and function. This could reflect an alternative regulatory mechanism to compensate for the loss of a functional second GR gene (Schaaf *et al.*, 2008).

Receptor functional definition by transactivation capacity. In carp, transactivation capacity of cortisol (capacity of hormone to initiate or repress CR-mediated transcription of down-stream genes) is about three-fold higher for GR2 (EC_{50} 2.4 ± 0.4 nM), than for GR1 (EC_{50} 7.1 ± 2.9 nM), and this would facilitate differential regulation by basal and elevated cortisol levels. The carp MR sensitivity (EC_{50} 4.1 ± 2.0 nM) is intermediate to that of the GRs, and this is in stark contrast to data for Burton's mouthbrooder (*Haplochromis burtoni*) and trout (*Oncorhynchus mykiss*), with MRs more sensitive to cortisol than the GRs (Greenwood *et al.*, 2003; Sturm *et al.*, 2005). In fish levels of up to 10 nM DOC were published (Campbell *et al.*, 1980). The low EC_{50} of DOC (0.25 nM) for MR transactivation in carp certainly does not exclude a mineralocorticoid function for DOC in fishes. In carp plasma basal total levels of cortisol are around 5 ng/ml (*i.e.* 13.8 nM); following stress, cortisol levels easily reach 150 ng/ml (> 400 nM). As only 20 % is available as unbound cortisol (Flik and Perry, 1989) this corresponds to 2.8 nM and > 80 nM, respectively, for which in carp apparently specific receptor subtypes are present: at rest both GR2 and MR may be occupied and activated, whereas GR1 is preferentially activated by stress-levels of

cortisol. In rodent brain a similar system was demonstrated: an 80 % MR and 10 % GR corticosterone occupancy was established in non-stressed situations (Reul *et al.*, 1987b). Even though the majority of MRs is occupied at low cortisol levels, it still is a dynamically regulated receptor. MR activity could be increased by an agonist to further inhibit HPA axis activity (Buckley *et al.*, 2007). We assume the same applies for common carp GR2 and MR. This means that although both receptors are partly or even largely occupied with cortisol, continuous modulation of HPI axis activity could be mediated by increasing cortisol levels after stressful events via GR1.

Receptor functional definition by localisation. As we focus on the involvement of the different receptors in stress axis regulation, our areas of interest are the HPI axis organs. Hypothalamus and pituitary gland of unstressed carp showed comparable mRNA levels for both GRs and the MR, suggesting functional importance of all three. Only in brain (without hypothalamus and pituitary gland), a consistent two-fold higher mRNA expression for GR1 over GR2 was seen. A receptor-defined duality in glucocorticoid function in brain of fishes seems of wider occurrence as similar preferential expression was seen in brain of rainbow trout and Burton's mouthbrooder (Bury *et al.*, 2003; Greenwood *et al.*, 2003). The higher mRNA expression level could translate into higher protein levels but may also reflect higher turnover. We have no data on brain cortisol levels but two receptors with significant difference in receptor sensitivity would allow differential responses to basal and stress levels of the steroid. In carp and trout (Sturm *et al.*, 2005), brain shows strong MR mRNA expression; unexpectedly, typical osmoregulatory organs such as gills, kidney and intestine show far lower MR mRNA expression. This suggests that a widely accepted but only presumed hypothesis of MR involvement in osmoregulation may be wrong, and is a consequence of extrapolation of the situation for aldosterone and MR function in mammals. However, also in the mammalian brain MRs play a key role in corticosteroid regulated processes (de Kloet *et al.*, 1998). The mammalian hippocampus exerts inhibitory control over HPA-axis activity: a dominant inhibition by MR activity is attenuated by GR activation (de Kloet *et al.*, 1998). Our results support such a combined function for steroids mediated by GR and MR activities in the regulation of stress coping and learning, and this is apparently a very early function developed in our ancestral fishes.

Steroid receptors distribution in telencephalic regions may contribute to define hippocampal regions, especially involved in (learning) behaviour and sentience. Our demonstration of GR and MR mRNA in carp telencephalon expands and further substantiates the notion that GR and MR corticosteroid receptors have basic functions in regulation of learning and memory. In goldfish (*Carassius auratus*) (Vargas *et al.*, 2006) and cod (*Gadus morhua*)

(Nilsson *et al.*, 2008) learning and spatial memory may involve the telencephalic lateral pallium, the homologue of the hippocampus of mammals (Meek and Nieuwenhuys, 1998).

In the hypothalamus, mRNA of all three receptor genes is expressed in parvo- and magnocellular neurons of the nucleus pre-opticus (NPO). We confirmed (data not shown) by immunohistochemistry on adjacent sections that expression of GR- and MR-mRNA co-localises with CRH (Huising *et al.*, 2004a). So far, the presence of GR protein in these cells was shown for rainbow trout (Teitsma *et al.*, 1998) and Mozambique tilapia (*Oreochromis mossambicus*) (Pepels *et al.*, 2004). However, the antiserum used in these studies does not distinguish between GR gene species. To the best of our knowledge, no data on MR distribution in other fish brains are available. In mammals the MR was described in the ventromedian and arcuate nuclei of the hypothalamus and the GR in the arcuate nuclei, paraventricular and supraoptic nucleus of the hypothalamus, (Reul and de Kloet, 1985); the hypothalamic arcuate nucleus is an important target for metabolic and hormonal signals controlling food intake and feeding behaviour (Meister, 2007). Clearly, future studies on GR and MR localisation in the fish analogues of these structures are warranted as we know that regulation of stress an feeding (arcuate nucleus in fish) are strongly intertwined, also in fish (Bernier *et al.*, 2004).

In carp pituitary gland, the two GR genes co-localise in the same areas. Remarkably little expression was seen in pars intermedia and would imply limited feedback by cortisol on MSH and somatolactin producing cells. This reminds of the situation reported for trout (Teitsma *et al.*, 1998), but not for tilapia that has significant GR-immunoreactivity in the pars intermedia (Pepels *et al.*, 2004), although mRNA expression could not be detected (Kitahashi *et al.*, 2007). The strongest signal for GR mRNA was seen in the proximal pars distalis GH-cells (in line with observations for GR1 on trout (Teitsma *et al.*, 1998) and tilapia; (Kitahashi *et al.*, 2007; Pepels *et al.*, 2004)). Stress/cortisol-induced suppression of growth, for instance seen after handling or confinement of fishes is thus explained by down-regulation of GH cells (Auperin *et al.*, 1997). In the rostral pars distalis finally, ACTH-cells express significant levels of GR mRNA, as one could predict as a basis for feedback control in the stress axis. Interestingly, recent data in tilapia showed no mRNA expression of either of the duplicated GRs in ACTH producing cells (Kitahashi *et al.*, 2007), whereas immuno-histochemical studies with an antibody against GR showed positivity in ACTH cells in tilapia and rainbow trout (Pepels *et al.*, 2004; Teitsma *et al.*, 1998). Prolactin cells, however, known to become activated during chronic stress (Auperin *et al.*, 1997), showed no detectable GR-expression (Teitsma *et al.*, 1998). This only seems at variance with recent reports on direct effects of cortisol on prolactin cells (Uchida *et al.*, 2004): a G-protein

coupled membrane receptor for cortisol should be considered. Indeed, the reported effects of cortisol on prolactin cells reported are fast, faster than one would predict for GR/MR mediated genomic effects.

MR mRNA was far more abundant in the pars distalis than in the pars intermedia. Based on comparisons with GR staining, we predict MR expression to be scarcely expressed in the α-MSH cells of the pars intermedia and strongly expressed in the GH cells of the pars distalis. Unfortunately, we were unable to demonstrate MR mRNA in ACTH cells specifically. Assuming (by analogy to the situation for GR mRNA) absence of MR mRNA in prolactin cells, our RQ-PCR data would favor presence of MR in ACTH-cells; this part of our study requires future attention.

3.4.2 Physiology

Restraint by netting for 24 hr resulted in hyperglycemia, hyponatremia, typical signs of strong catecholaminergic activation and inherent loss of control over integumental permeability to water and ions (Wendelaar Bonga, 1997). A persistent hypercortisolinemia was observed, but the duration of the stress condition proved insufficient to induce feedback by cortisol. However, a more persistent stress of repeated cold transfer induced high cortisol levels and hyperglycemia and mild hypernatremia indicative of adaption to the stressor. Mild hypernatremia is typical for enhanced prolactin activity and successful restoration of blood mineral levels to a new set point (Metz *et al.*, 2003). This longer-term adaptation process did involve measurable down-regulation of CRs in brain (without hypothalamus and pituitary gland). This observation suggests a central initiation of downregulation of cortisol release, rather than direct feedback via NPO or pituitary gland. In tilapia, a similar regulation system was suggested; cortisol feedback on CRH release is exerted via the forebrain medial part of dorsal telencephalon (Pepels *et al.*, 2004). Our observation of GR and MR mRNA down-regulation only following persistent stress is further consistent with experiments on Coho salmon (*Oncorhynchus kisutch*) that showed no effect of acute stress on GR expression whereas chronically elevated cortisol levels in several other paradigms did (Maule and Schreck, 1991).

The MR mRNA in carp brain was downregulated to the same extent as the mRNA levels of both GRs. This seems in contrast with the mammalian model where GR stimulates HPA axis activity, whereas MR has an inhibiting effect (de Kloet *et al.*, 1998). However, a similar result was found in hippocampus of rats exposed to increasing corticosteroid levels (Hugin-Flores *et al.*, 2004). And in mouse pups with high corticosteroid levels due to 24 hr maternal deprivation, both GR and MR mRNA expression were significantly decreased

(Schmidt *et al.*, 2003). These discrepancies between different stressors are thus of wider occurrence and require further and species specific approaches.

In conclusion, we show that carp express separate glucocorticoid receptors for regulation under basal and stressful conditions. We predict an ancestral role not only for the duplicated GRs but also for MR in stress physiology, based on receptor localisation and expression profile after chronic stress and provide a receptor profile in brain conform memory and learning functionalities in fishes as in higher vertebrates. A role for DOC in stress physiology in fishes has never been considered but seems a reasonable hypothesis for future research. More in-depth studies on specific roles of these receptors in learning and stress physiology will therefore elucidate early strategies in vertebrate evolution to control the basics of life.

Ackowledgements

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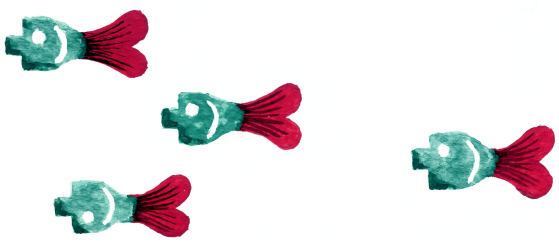

'Yet where they are, and how many, and what they will do next, we do not know.'

J.R.R. Tolkien, The lord of the rings

4

Stress and innate immunity in carp: corticosteroid receptors and pro-inflammatory cytokines

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[1]

Abstract

The stress hormone cortisol is deeply involved in immune regulation in all vertebrates. Common carp (*Cyprinus carpio* L.) express four corticoid receptors that may modulate immune responses: three glucocorticoid receptors (GR); GR1, with two splice variants, (GR1a and GR1b), GR2 and a single mineralocorticoid receptor (MR). All receptors are expressed as of 4 days post-fertilisation and may thus play a critical role in development and functioning of the adult immune system. Immune tissues and cells predominantly express mRNA for GRs compared to mRNA for the MR. Three-dimensional protein structure modelling predicts, and transfection assays confirm that alternative splicing of GR1 does not influence the capacity to induce transcription of effector genes. When tested for cortisol activation, GR2 is the most sensitive corticoid receptor in carp, followed by the MR and GR1a and GR1b. Lipopolysaccharide (LPS) treatment of head kidney phagocytes quickly induces GR1 expression and inhibits GR2 expression. Cortisol treatment *in vivo* enhances GR1a and MR mRNA expression, but only mildly, and cortisol-treatment *in vitro* does not affect receptor expression of phagocytes. Cortisol has no direct effect on the LPS-induced receptor profile. Therefore, an immune rather than a stress stimulus regulates GR expression. Cortisol administered at stress levels to phagocytes *in vitro* significantly inhibits LPS-induced expression of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12) (subunit p35) and of inducible nitric oxide synthase (*i*NOS) expression. A physiologically differential function for GR1 and GR2 in the immune response of fish to infection is indicated.

4.1 Introduction

In teleostean fishes, such as carp, cortisol regulates a plethora of physiological processes such as development, reproduction, osmoregulation, metabolism, and immunity. Cortisol is the dominant steroid in stress physiology (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997) and has profound and differential effects on the immune system. Stress often inhibits immune responses, to counteract potential deleterious effects of too strong pro-inflammatory responses; acute stress and related cortisol release may exert stimulatory effects (Butts and Sternberg, 2008; Elenkov and Chrousos, 2006; Fast *et al.*, 2008). Indeed, in common carp, stress reduces the number of circulating B-lymphocytes, and decreases the antibody response after immunisation *in vivo* (Verburg-van Kemenade *et al.*, 1999); cortisol inhibits inflammatory cytokine expression *in vitro* (Saeij *et al.*, 2003c). Cortisol and lipopolysaccharide (LPS) synergistically stimulate the expression of interleukin 1β (IL-1β) mRNA in head kidney phagocytes (Engelsma *et al.*, 2003b). Interestingly, stress can have opposite effects. Cortisol stimulates apoptosis of B-cells (Weyts *et al.*, 1998a), and inhibits apoptosis of neutrophils (Weyts *et al.*, 1998b), an adaptive response to prolong the life span of neutrophils that form the first-line defence against pathogens.

Production in fish aquaculture is increasing rapidly over the last decennia. Stress as a result of high rearing density and handling gives rise to immune suppression and increased susceptibility to infectious diseases, especially in larvae and juveniles (Maule and Schreck, 1991; Palermo *et al.*, 2008; Terova *et al.*, 2005). To steer disease prevention we need better understanding of mechanisms of stress-induced modulation of immunity. In teleostean fishes, the stress hormone cortisol is produced following activation of the hypothalamo-pituitary-interrenal (HPI) axis, the functional analogue of the mammalian hypothalamo-pituitary-adrenal (HPA) axis. The hydrophobic cortisol enters the cell and activates cytosolic transcription factors, such as the glucocorticoid receptor. The hormone receptor complex translocates to the nucleus and binds to specific glucocorticoid responsive elements (GREs) in the DNA, to activate or repress transcription of genes (Kumar and Thompson, 2005). Formation of the ligand-receptor complex and transactivation or transrepression is hypothesised to utilise similar intracellular routes in teleostean fishes as in mammals (Stolte *et al.*, 2006).

Teleostean fishes express more corticoid receptors than other vertebrates. Although fish do not produce aldosterone (Jiang *et al.*, 1998), they do express mRNA and the protein of a mineralocorticoid receptor (MR), which can bind cortisol. Fish do produce significant amounts of 11-deoxycorticosterone (DOC) that may serve a role as mineralocorticoid, but

physiological data on for instance plasma levels of this steroid in fishes are very scarce and await further studies (Prunet *et al.*, 2006; Sturm *et al.*, 2005). Furthermore, fishes have duplicate GR genes (GR1 and GR2) that both transcribe into functional proteins (Bury *et al.*, 2003). Moreover, the GR1 gene may yield two splice variants, as was demonstrated for rainbow trout (*Oncorhynchus mykiss*). (Lethimonier *et al.*, 2002; Takeo *et al.*, 1996) and two pufferfish species (Stolte *et al.*, 2006). Both splice variants are constitutively expressed and induce transcription (Greenwood *et al.*, 2003; Takeo *et al.*, 1996). Interestingly, the duplicated receptors require different concentrations of cortisol (low and high, basal and stress levels) to initiate transcription in effector cells (transactivation capacity) (Bury *et al.*, 2003; Greenwood *et al.*, 2003). This notion opens a vast range of opportunities for differential regulation with a single ligand, viz. cortisol.

The role of corticoid receptor subtypes in immune modulation was investigated. Corticoid receptor expression during early development suggests that stress or cortisol can affect the developing immune system. Based on constitutive mRNA expression levels and sensitivity for cortisol we show that the glucocorticoid receptors rather than the MR are important in immune regulation. GR expression is differentially adjusted following LPS treatment. We show inhibition of mRNA expression of the pro-inflammatory inducible nitric oxide synthase (*i*NOS) and pro-inflammatory cytokines by stress levels of cortisol. The results widen our insight into the intricate, cortisol-induced, immune modulation.

4.2 Experimental procedures

4.2.1 Animals

Common carp (*Cyprinus carpio* L.) were kept at 23 °C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7 % of their estimated body weight. The cross 'R3×R8' is offspring of Hungarian (R8) and Polish (R3) strains (Irnazarow, 1995). Experimental repeats were performed with fish reared from different batches of eggs. All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

4.2.2 Tissue preparation

Nine month old carp (150–200 g) were anaesthetised with 0.2 g/l tricaine methane sulfonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l $NaHCO_3$

(Merck, Darmstadt, F.R. Germany). Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe fitted with a 21 gauge needle. Next fish were killed by spinal transection and organs and tissues were carefully removed, snap frozen in solid CO_2 or liquid N_2 and stored at -80 °C for RNA extraction. Whole carp embryos were anaesthetised with 0.2 g/l TMS buffered with 0.4 g/l $NaHCO_3$ at the indicated stages of development. Individual eggs or embryos were snap frozen in liquid N_2 and stored at -80 °C.

4.2.3 Restraint-stress paradigm

Prolonged restraint (24 hr) was given by netting the fish and suspending the nets with the fish in the tanks (Huising *et al.*, 2004a). After 24 hr, the experimental group was transferred at once to a tank with 0.2 g/l TMS, resulting in rapid ($<$ 1 min) and deep anaesthesia prior to blood sampling and killing. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anaesthesia, immediately before sampling of the experimental group. Blood and organs were isolated as mentioned above.

4.2.4 Plasma hormone determination

Freshly collected, heparinised blood was centrifuged for 10 min at 2000 *g* at 4 °C, after which plasma was transferred to a new tube and stored at -20 °C. Cortisol was measured by RIA (Arends *et al.*, 1998) with a commercial antiserum (Bioclinical Services Ltd, Cardiff, UK). All constituents were in phosphate-EDTA buffer (0.05 M Na_2HPO_4, 0.01 M Na_2EDTA, 0.003 M NaN_3, pH 7.4). Ten-microliter samples or standards in RIA buffer (phosphated-EDTA buffer containing 0.1 % 8-anilia-1-naphthalene sulphonic acid and 0.1 % w/v bovine γ-globulin) were incubated with 100 µl antiserum (in RIA buffer containing 0.2 % normal rabbit serum) for 4 hr. Samples were incubated overnight with 100 µl iodinated cortisol (around 1700 cpm/tube; ^{125}I-labelled cortisol, Amersham; Uppsala, Sweden) and 100 µl goat anti-rabbit γ-globulin (in RIA buffer). Bound and free cortisol in the assay were separated by the addition of 1 ml ice-cold precipitation buffer (phosphate-EDTA buffer containing 2 % w/v bovine serum albumin and 5 % w/v polyethylene glycol). Tubes were centrifuged at 4 °C (20 min at 2000 *g*), the supernatant aspirated and the pellets counted in a gamma counter (1272 clinigamma, LKB, Turku, Finland).

4.2.5 Cell culture

Anterior head kidney phagocytes were obtained by passing the tissue through a 100 µm nylon mesh (BD Bioscience, Breda, The Netherlands) with carp RPMI (cRPMI; RPMI 1640, Invitrogen Carlsbad, CA, USA; adjusted to carp osmolality (280 mOsm/kg) and washed twice. The cell suspension was layered on a discontinuous Percoll (Amersham, Biosciences, Uppsala, Sweden) gradient (1.020, 1.060, 1.070 and 1.083 g/cm^3) and centrifuged 30 min at 800 *g* with the brake disengaged. Cells at the 1.070 (65 % macrophages,10 % granulocytes and 25 % small macrophages and lymphocytes) and 1.083 g/cm^3 (85 % neutrophilic granulocytes, 15 % macrophages) interface (van Kemenade *et al.*, 1994) were collected and washed twice with cRPMI and once with cRPMI^{++} (cRPMI supplemented with 0.5 % pooled carp serum, 1 % glutamine (Cambrex, Verviers, Belgium), 1 % penicillin G (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1 % streptomycin sulphate (Sigma-Aldrich)). Subsequently treatments were carried out *in duplo* in cRPMI^{++} at 5.5×10^6 cells per well (in 500 µl) in a 24-well cell culture plate. Cells were stimulated for 4 hr at 27 °C at 5 % CO_2 with 30 µg/ml LPS (Lipopolysaccharide from *Escherichia coli 055:B5*, Sigma-Aldrich), or with 100 nM cortisol (Sigma-Aldrich) or a combination of both. Control cells received medium only and experiments were repeated for four independent fish. After stimulation, supernatant was removed and cells were collected in 300 µl RLT buffer from the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and stored at -80 °C (duplicate treatments were pooled).

4.2.6 RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen). Total RNA was precipitated in isopropanol, washed with 75 % ethanol and dissolved in nuclease-free water. RNA of cells was isolated as described by the RNeasy Mini Kit (Qiagen) strictly according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry and integrity was ensured following electrophoresis in a 1.5 % agarose gel before proceeding with cDNA synthesis.

4.2.7 DNAse treatment and first strand cDNA synthesis

For each sample a '–RT' (non reverse transcriptase) control was included. One microliter of 10× DNase-I reaction buffer and 1 µl DNase-I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65 °C for 10 min. To each sample, 300 ng

random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5× First Strand buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (DTT) and 40 Units RNAse Out (Invitrogen 10777-019) were added and the mix was incubated for 10 min at room temperature and for an additional 2 min at 37 °C. To each sample (not to the '–RT' controls) 200 U Superscript-II RNase H^- Reverse Transcriptase (RT; Invitrogen, 18064-014) was added and reactions were incubated for 50 min at 37 °C. Demineralised water was added to a final volume of 100 µl and samples were stored at -20 °C until further use.

Table 4.1: Primers used for gene expression studies. Table shows sequences and GenBank accession numbers.

Gene	FW primers	RV primers	Acc. No.
GR1a	GTTCGTCGGATCAGCAAGC	CTGCGTTTTGTCGTGCTCTC	AJ879149
GR1b	GTTCGTCGGATCAGCAAGC	TTGTGCTGCCCCTCTACG	AM697886
GR2	GGAGAACAACGGTGGGACTAAAT	GGCTGGTCCCGATTAGGAA	AM183668
MR	TTCCCTGCAGAACTCAAAGGA	ACGGACGGTGACAGAAACG	AJ783704
IL-10	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	AJ245635
TNF-α 1, 2	GCTGTCTGCTTCACGCTCAA	CCTTGGAAGTGACATTTGCTTTT	AJ311800
*i*NOS	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCAGAGTCTCTTCT	AJ242906
p35	TGCTTCTCTGTCTCTGTGATGGA	CACAGCTGCAGTCGTTCTTGA	AJ580354
p40a	GAGCGCATCAACCTGACCAT	AGGATCGTGGATATGTGACCTCTAC	AJ621425
CXCa	CTGGGATTCCTGACCATTGGT	GTTGGCTCTCTGTTTCAATGCA	AJ421443
CXCb	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB082985
CXCR1	GCAAATTGGTTAGCCTGGTGA	AGGCGACTCCACTGCACAA	AB010468
CXCR2	TATGTGCAAACTGATTTCAGGCTTAC	GCACACACTATACCAACCAGATGG	AB010713
IL-10	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT	AB110780
TGF-β	ACGCTTTATTCCCAACCAAA	GAAATCCTTGCTCTGCCTCA	AAF22573
β-Actin	GCTATGTGGCTCTTGACTTCGA	CCGTCAGGCAGCTCATAGCT	M24113
40S	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087

4.2.8 Real-time quantitative PCR

PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and *PRIMER3* software (Rozen and Skaletsky, 2000) was used to design primers for use in real-time quantitative PCR (RQ-PCR) (table 4.1). For RQ-PCR 5 µl cDNA and forward and reverse primers (300 nM each) were added to 7 µl Brilliant® SYBR® QPCR Master Mix (Stratagene, La Jolla, CA, USA) and demineralised water was added to a final volume of 14 µl. RQ-PCR (10 min 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C followed by 1 min at 60 °C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed with comparative quantitation of the Rotor-gene Analysis Software V5.0. Basal gene expression in organs and tissues was determined as a ratio

of target gene *vs* reference gene and was calculated according to the following equation.

$$\text{ratio} = \frac{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}}}{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}}} \tag{4.1}$$

where E is the amplification efficiency and Ct is the number of PCR-cycles needed for the signal to exceed a predetermined threshold value. Expression following *in vitro* treatment was determined relative to the expression of non-restraint control fish according to the following equation (Pfaffl, 2001).

$$\text{ratio} = \frac{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}(\text{control} - \text{sample})}}{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}(\text{control} - \text{sample})}} \tag{4.2}$$

Two internal reference genes (40S ribosomal protein and β-actin) were incorporated in all RQ-PCR experiments; results were similar following standardisation to either gene. '–RT'-controls were included in all experiments and no amplification above background levels was observed. Non-template controls were included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked by sequencing.

4.2.9 Transactivation assay

The clones encoding full-length open reading frame of common carp GR1a and GR1b were excised from pGEM-Teasy vector by EcoR1 and BamH1 and ligated into pcDNA3 expression vector, cut with the same enzymes. Orientation and quality of the insert was confirmed by sequencing. COS-7 cells (derived from African green monkey kidney) were cultured as described previously (Sturm *et al.*, 2005). Cells were transiently transfected using a calcium precipitation method (Sambrook and Russell, 2001), with cells growing in the log phase at 30–50 % confluence. Cells were co-transfected with the following plasmids: expression vector with the appropriate hormone receptor cDNA, (1 µg / 24 well plate), reporter plasmid pFC31Luc, which contains the mouse mammary tumour virus promoter upstream of the luciferase gene (MMTV-LUC) (10 µg / 24 well plate), and pSVβ (Clontech, Palo Alto, CA, USA), a second reporter plasmid under control of the SV40 promoter and serving as a control for transfection efficiency (2 µg / 24 well plate) and finally pBluescript (Clontech, Palo Alto, CA, USA) (7 µg / 24 well plate), an irrelevant plasmid to increase transfection. Sixteen hours after transfection, medium was renewed and cortisol added from 1000 fold concentrated stock solution in ethanol. After 36 hr incubation, cells were harvested using

reporter lysis buffer (Promega, Madison, WI, USA) following the manufacturer's instructions and luciferase and β-galactosidase activities were determined as described previously (Bury *et al.*, 2003). In addition to solvent controls (receiving only ethanol carrier instead of hormone) cells were transfected with an empty expression vector to control for luciferase activity in absence of hormone receptor DNA. Experiments were repeated three times independently with triplicate cell cultures per treatment. Luciferase activity was corrected for 'well-specific' transfection efficiency (determined by β-galactosidase activity) and then expressed as percentage luciferase activity observed in cells treated with 10^{-7} M cortisol. Kinetic parameters (maximum velocity, V_{max} and half maximum activation concentration (EC_{50}) in the transactivation assay were assessed by fitting the data to a single ligand binding model using SigmaPlot® software. Only converging data sets were included in data sets presented. Ligands were tested in the range of 10 pM to 1 μM. Data were normalised to maximal (100 %) response and corrected for blanks prior to kinetic analysis.

4.2.10 Molecular modelling

Homology modelling techniques were used to construct a model of the carp glucocorticoid receptor (GR) DNA-binding domain (DBD). A crystal structure of the rat GR DBD in complex with DNA, solved at 2.5 Å resolution resolution (Luisi *et al.*, 1991), was used as a modelling template (Protein Data Bank (Berman *et al.*, 2000) ID: 1R4O). Aside from the nine additional amino acids in the carp DBD, the sequences of the two DNA binding domains are virtually identical (98 % sequence identity). The nine inserted amino acids were modelled using YASARA (http://www.yasara.org) by searching a non-redundant subset of the DBD for loops with similar sequence and start and end anchor points. Subsequently, side chains were modelled and the model was optimised using the Yamber2 force field to accommodate the changes (Krieger *et al.*, 2004). A coordinate file of the model is available from the authors upon request.

4.2.11 Statistics

Statistical analyses were performed using SPSS 12.0.1 software. Differences in corticosteroid receptor expression were evaluated with a Student's *t*-test and $P < 0.05$ was accepted as fiducial limit. Homogeneity was tested with Levene's test and we corrected the Student's *t*-test for unequal variances when necessary. In case of RQ-PCR data, tests were performed for both internal reference genes (β-actin and 40S) and statistical significance was only reported if both reference genes showed a significant effect. Cytokine expression and EC_{50} of

receptors were compared and differences tested with a Mann-Whitney U test, and $P < 0.05$ was accepted as significant. Data are represented as average and error bars indicate standard deviation, * indicates $P < 0.05$, ** indicates $P < 0.01$.

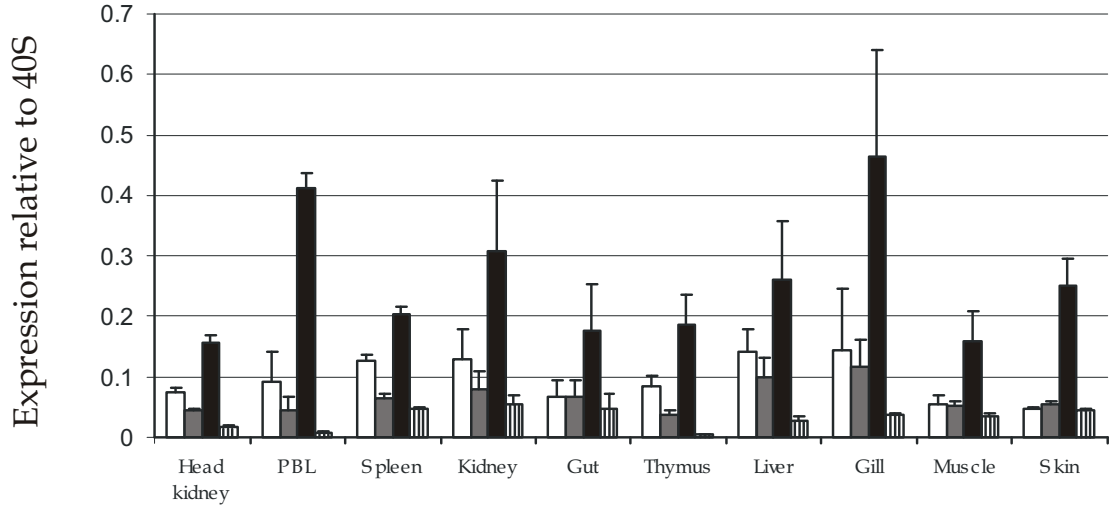
4.3 Results

Widespread mRNA expression of corticoid receptors in immune organs. GR1a, GR1b, GR2, and MR genes are constitutively expressed in all immune tissues and tissues rich in immune cells (epithelia of gills, skin gut en kidney) (figure 4.1a). The expression levels were comparable to those found in brain, hypothalamus and pituitary, (Stolte *et al.*, 2008a), reported recently for the same species. Messenger RNA levels for the GR2 were consistently the highest, those for both GR1a and GR1b being about half this level and that for MR was on average less than 30 % of GR2 expression. Gills and peripheral blood lymphocytes (PBL) showed the highest relative GR2 expression. In most organs tested GR1a expression was slightly higher than GR1b expression. MR expression levels were especially low in immune tissues; thymus, PBL, and head kidney.

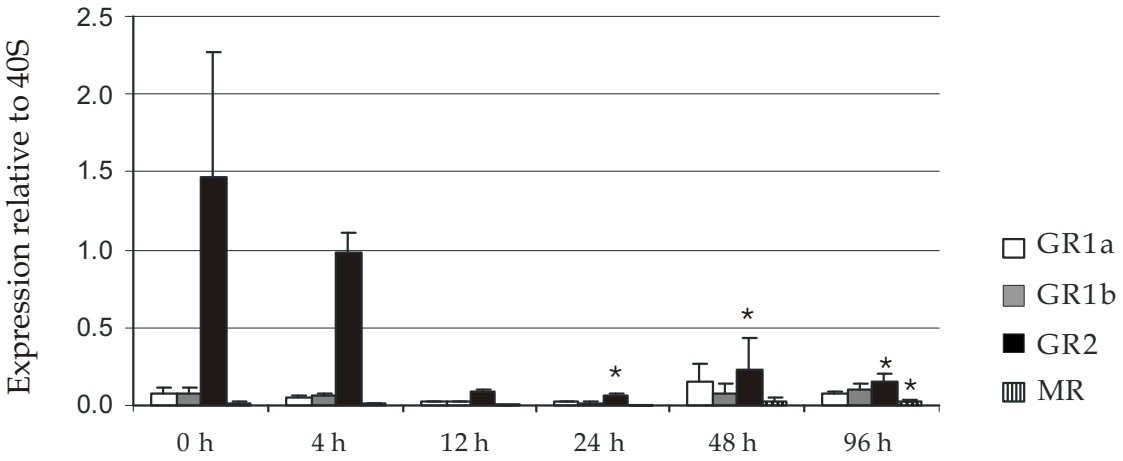
During early development GR1 (a and b) mRNA expression levels in whole embryos were comparable to messenger RNA levels in separate organs of adult fish (figure 4.1b). In unfertilised eggs and embryos of 4 hr post-fertilisation (4 hpf) GR2 mRNA expression was ten-fold stronger than expression of either GR1 gene. These GR2 mRNA levels quickly and dramatically dropped until 24 hpf, after which levels remained constant. MR was hardly expressed until 24 hpf, but at 48 hpf expression levels started to rise, and at 96 hpf, MR mRNA expression levels were significantly increased compared to unfertilised eggs and 50 % compared to expression of either GR1 gene.

Homology modelling of the fish specific DNA binding domain. As the carp GR1a has a nine amino acid insert (WRARQNADG) in the very conserved DNA binding region (figure 4.2a and 2b), we first modelled the receptor DNA binding site. We constructed a protein model of the carp GR1a DNA binding domain based on the rat GR DNA binding domain crystal structure (figure 4.2c), and used the YASARA program to predict the three-dimensional structure of the nine amino acid insert. The nine amino acid insert extends the loop between the two zinc fingers and projects outward, away from the DNA (figure 4.2d). The model demonstrates that the insert does not necessarily disturb the zinc finger residues involved in DNA binding.

GR1a and GR1b transactivation. GR1a and GR1b showed similar affinities for the different hormones tested (figure 4.3); dexamethasone was the strongest agonist tested, followed



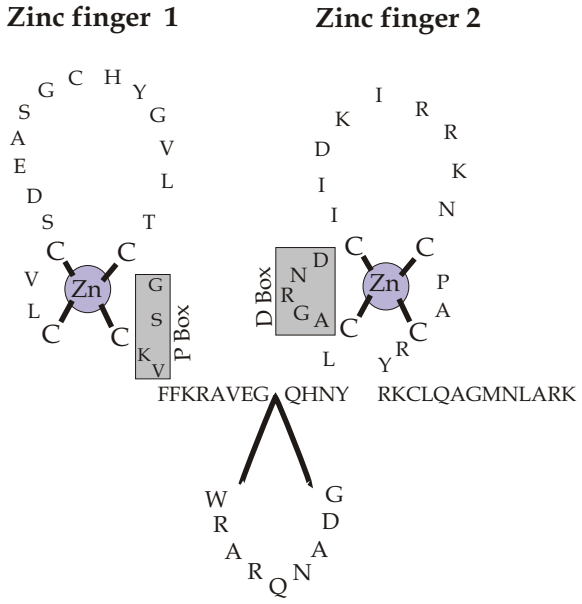
(a) Constitutive corticoid receptor expression



(b) Corticoid receptor expression during early ontogeny

Figure 4.1: Constitutive corticoid receptor mRNA expression in peripheral organs (a). cDNA of different organs, or freshly isolated blood lymphocytes, was used as template for quantitative real time PCR. Messenger RNA expression data of four control fish is shown relative to the housekeeping gene 40S. Corticoid receptor mRNA expression during early ontogeny (b). cDNA of four or five individual eggs or embryos (0, 4, 12, 24, 48 and 96 hpf) was used as template for quantitative real time PCR. Messenger RNA expression data is shown relative to the housekeeping gene 40S. * indicates $P < 0.05$.

by cortisol, deoxycortisol and corticosterone. Aldosterone and DOC, were very weak agonists. The physiologically important stress hormone cortisol was chosen as ligand to compare sensitivity between the two splice variants. EC_{50}-values were found to be comparable; 7.1 ± 5.0 nM for GR1a and 17.4 ± 7.5 nM for GR1b, concentrations compatible with basal plasma cortisol levels.



(a) Alternative splicing in GR1

```
hGR     CLVCSDEASGCHYGVLTCGSCKVFFKRAVEG---------QHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQAGMN
cGR1a   CLVCSDEASGCHYGVLTCGSCKVFFKRAVEGWRARQNADGQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGMN
cGR1b   CLVCSDEASGCHYGVLTCGSCKVFFKRAVEG---------QHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGMN
        ********************************         **************************:*********
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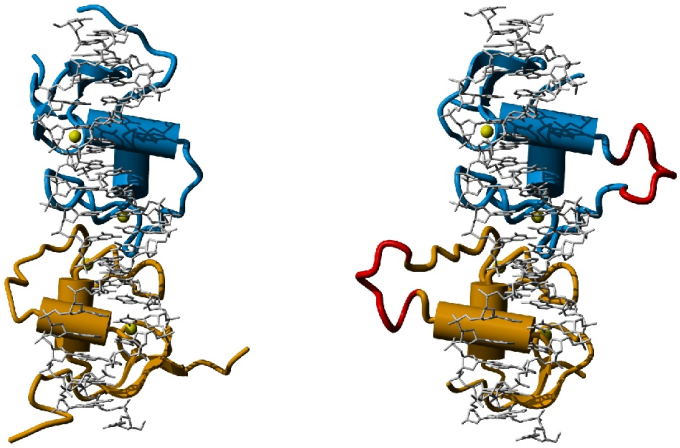
(b) GR alignment



(c) Rat GR (d) Carp GR1a

Figure 4.2: Alternative splicing in the DNA binding domain of GR1. Schematic representation of the two zinc fingers in the GR DNA binding domain and location of insertion of 9 amino acids as a result of alternative splicing (a). Sequence alignment of rat GR (acc. number NP_036708) and common carp GR1a and GR1b (b). Identical amino acids are indicated by *, and amino acids with high similarity are indicated as :. Protein modelling of GR DNA binding region (c) and (d). DNA binding domain of carp GR1a (with 9 amino acid insert (d)) is modelled over the rat GR crystal structure (c)(PDB **ID: 1R4O**). Wire frame represents DNA, ribbon and pipe structures represent two GR DNA binding regions that form a homodimer. Single balls represent zinc atoms of the zinc fingers. The 9 amino acid insert of common carp in each of the two DNA binding regions is indicated.

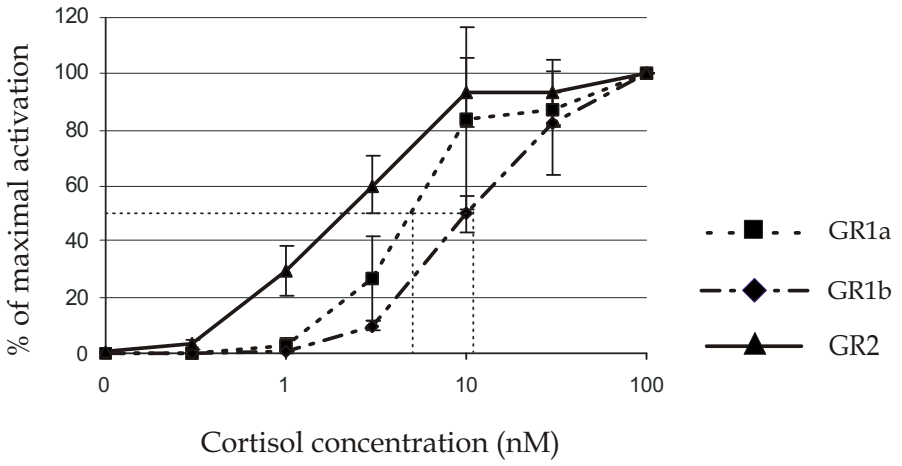
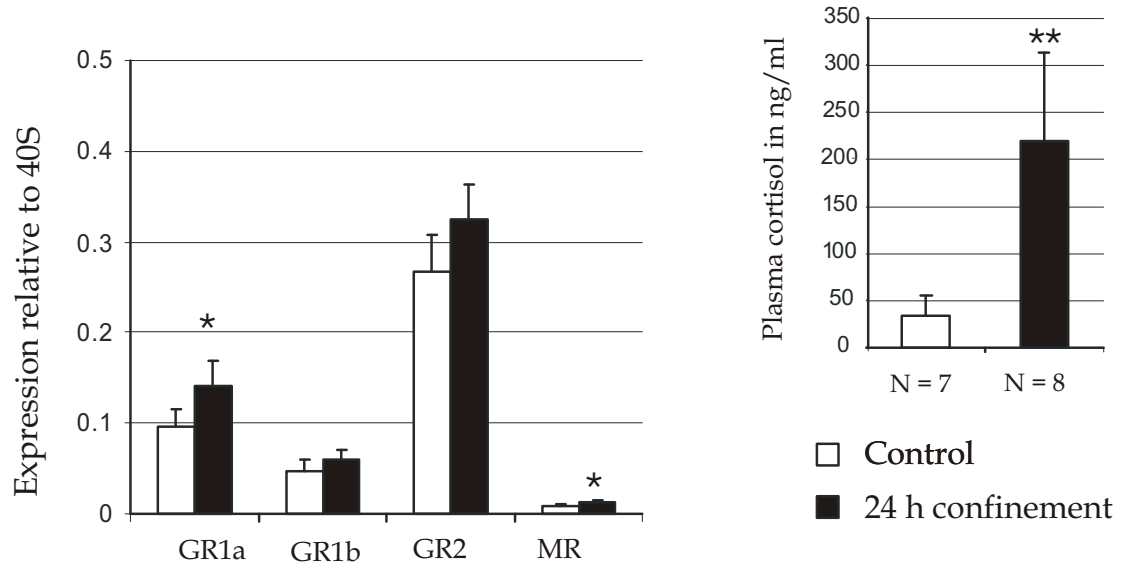


Figure 4.3: Transactivation properties of glucocorticoid receptors in response to cortisol. COS7 cells were co-transfected with either GR1a (squares), GR1b (diamonds) or GR2 (triangles) expression vectors, together with reporter plasmid pFC31 Luciferase (under control of a MMTV promoter) and the pSVβ plasmid, which expresses β-galactosidase. After transfection cells were treated with varying (0.1 to 1000 nM) cortisol concentrations. Transactivation was determined by luciferase activity normalised to the internal β-galactosidase control. Data are expressed as percent activity of 100 nM cortisol and represent the average of three separate experiments. EC_{50} is indicated by the dotted line.

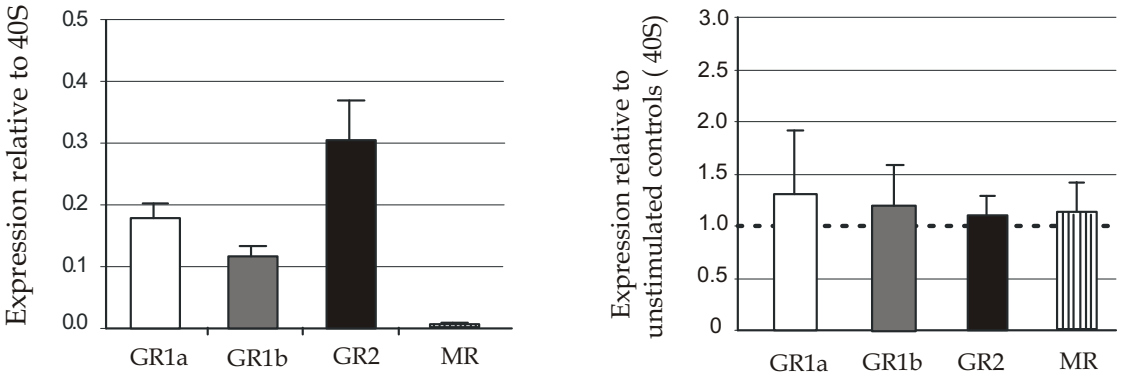
Stress only mildly increases corticosteroid mRNA expression in head kidney. The effect of stress on corticoid receptor expression was determined in head kidney in head kidney *in vivo* and in head kidney phagocytes *in vitro*. In an *in vivo* experiment, 4 fish were confined in a net for 24 hr, which resulted in acute stress, reflected by significantly increased plasma cortisol levels (figure 4.4a). After 24 hr the head kidneys were removed and corticoid receptor mRNA expression was determined. GR1a and MR expression was slightly, but significantly increased; expression of GR1b and GR2 mRNA did not change significantly.

In a separate experiment the effect of 100 nM cortisol-treatment on corticoid receptor expression in head kidney phagocytes *in vitro* was determined (figure 4.4b). Constitutive GR1 (a and b) mRNA expression was higher and constitutive MR expression was lower in head kidney phagocytes than in whole head kidney tissue. Cortisol did not significantly affect GR1 (a and b), GR2 and MR mRNA expression.

LPS treatment increases GR1a and decreases GR2 expression in head kidney phagocytes. Head kidney phagocytes were stimulated with 50 μg/ml LPS (figure 4.5a). GR1a expression was doubled after 2 hr and was still increased after 4 hr; GR2 expression was decreased at both 2 hr and 4 hr. As immune stimuli often associate with a stress response, we determined *in vitro* the effect of a sub-optimal LPS-stimulation (30 μg/ml) alone or in combination with 100 nM cortisol on corticoid receptor expression in head kidney phagocytes (figure 4.5b).

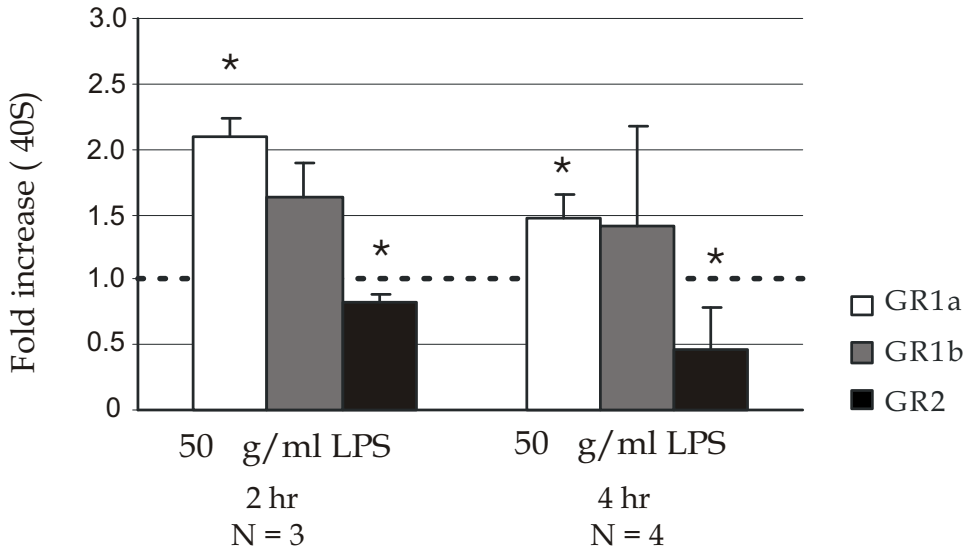


(a) Whole head kidney

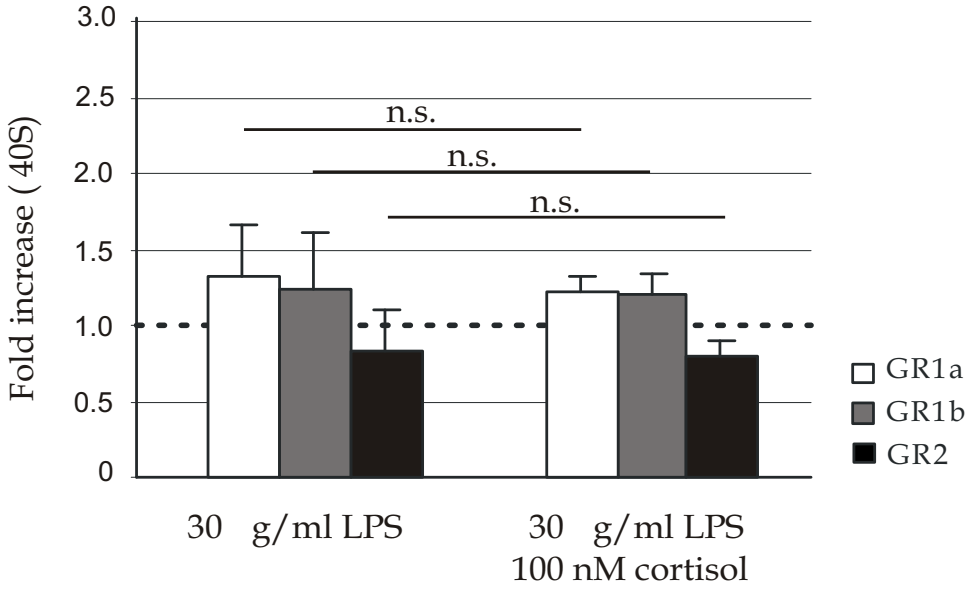


(b) Head kidney phagocytes

Figure 4.4: Corticoid receptor mRNA expression after acute stress or *in vitro* cortisol-treatment. After 24 hr of confinement by netting, head kidneys from control or confined animals were used as template for real time quantitative PCR (a). Messenger RNA expression data of four fish per group are shown relative to the housekeeping gene 40S. Plasma cortisol levels of control and confined animals are shown in insert. After 4 hr *in vitro* treatment with 100 nM cortisol head kidney phagocytes were used as template for real time quantitative PCR. Constitutive mRNA expression in head kidney phagocytes of four control fish is shown relative to the housekeeping gene 40S (b left). Messenger RNA expression after cortisol-treatment is shown as ×-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S (b right). * indicates $P < 0.05$.



(a) 50 µg LPS treatment



(b) 30 µg LPS treatment

Figure 4.5: LPS-induced glucocorticoid receptor expression. Freshly isolated head kidney phagocytes were stimulated for 2 or 4 hr with 50 µg/ml LPS (a). Messenger RNA expression data of four control fish is shown as ×-fold increase compared to unstimulated control cells (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S. Constitutive expression of control cells relative to the housekeeping gene 40S; 2 hr treatment: GR1a: 0.022 ± 0.011, GR1b: 0.021 ± 0.010, GR2: 0.078 ± 0.036, 4 hr treatment: GR1a: 0.028 ± 0.011, GR1b: 0.033 ± 0.018, GR2: 0.106 ± 0.005. Glucocorticoid receptor expression after LPS or LPS and cortisol-treatment (b). Freshly isolated head kidney phagocytes were stimulated for 4 hr with 30 µg/ml LPS or with 30 µg/ml LPS and 100 nM cortisol. Messenger RNA expression data of four control fish are shown as ×-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S. Constitutive expression of control cells relative to the housekeeping gene 40S; GR1a: 0.177 ± 0.025, GR1b: 0.118 ± 0.017, GR2: 0.306 ± 0.063. * indicates $P < 0.05$. N.S. not significant

Also, after 4 hr of treatment with 30 μg/ml LPS, GR1 (a and b) mRNA expression was increased, while GR2 expression was decreased. After addition of cortisol to these LPS-treated phagocytes, glucocorticoid receptor mRNA expression levels did not differ from expression levels after treatment with LPS only.

Cortisol inhibits LPS-induced iNOS mRNA expression. To mimick stress-induced immune modulation *in vitro*, the effect of cortisol exposure on head kidney phagocyte *i*NOS production in response to an immune stimulus (LPS) was investigated (figure 4.6). Compared to control cells, addition of 1 nM cortisol did not affect *i*NOS expression. Addition of 100 nM cortisol slightly decreased *i*NOS expression. Treatment with 30 μg/ml LPS increased *i*NOS expression 75 fold (12 to 200 fold), (table 4.2). Addition of 1 nM (only GR2 activated) of cortisol did not affect LPS-induced *i*NOS expression, 100 nM cortisol (all GRs maximally activated) significantly decreased LPS-induced *i*NOS expression.

Table 4.2: Cytokine mRNA expression of non-stimulated and LPS-stimulated head kidney phagocytes. Cells were stimulated for 4 hr with 30 μg/ml LPS. Constitutive and LPS-induced cytokine expression of four separate *in vitro* experiments as measured by quantitative real time PCR is shown relative to housekeeping gene 40S. Average fold increase in cytokine expression as result of LPS treatment is mentioned. * represents $P < 0.05$.

Gene	Constitutive expression	LPS-stimulated expression	Fold increase	
IL-12 p35	$6.69 \pm 5.44 \times 10^{-5}$	$5.13 \pm 4.97 \times 10^{-4}$	7	*
IL-12 p40	$1.88 \pm 1.28 \times 10^{-4}$	$2.07 \pm 4.67 \times 10^{-4}$	150	*
CxCb	0.001 ± 0.001	0.002 ± 0.001	1	
IL-10	0.004 ± 0.002	0.014 ± 0.007	4	*
*i*NOS	0.046 ± 0.048	1.030 ± 0.331	75	*
CxCR2	0.061 ± 0.034	0.077 ± 0.055	1	
TNF-α	0.118 ± 0.119	0.350 ± 0.288	3	*
TGF-β	0.212 ± 0.412	0.201 ± 0.376	1	
CxCa	0.485 ± 0.180	0.422 ± 0.162	1	
IL-1β	0.517 ± 0.320	3.436 ± 0.998	7	*
CxCR1	0.709 ± 0.160	1.538 ± 0.328	2	

Cortisol inhibits LPS-induced cytokine mRNA expression. Treatment with 30 μg/ml LPS significantly induced tumor necrosis factor alpha (TNF-α), IL-1β, interleukin 12 subunits p35 and p40 and interleukin 10 (IL-10) mRNA expression (table 4.2). The same stimulus did not significantly affect mRNA expression levels of chemokines CxCa and CxCb, chemokine receptors CxCR1, CxCR2, and transforming growth factor beta (TGF-β) (table 4.2). Addition of 100 nM of cortisol significantly inhibited LPS-induced upregulation of tumor necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) subunit p35 mRNA expression

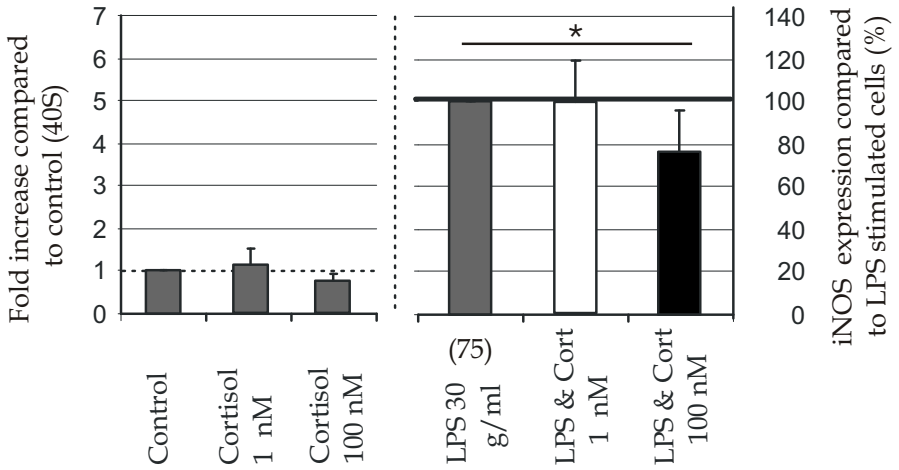


Figure 4.6: *i*NOS mRNA expression in stimulated head kidney phagocytes. Freshly isolated head kidney phagocytes were stimulated for 4 hr with either 1 nM or 100 nM cortisol, 30 µg/ml LPS or a combination of cortisol and LPS. Messenger RNA expression data of four control fish are shown as ×-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S (**left**). Messenger RNA expression data of four control fish are shown as percentage of LPS-induced expression (set at 100 %, represented by the thick line) (**right**). Average increase of mRNA expression after LPS treatment is shown in (brackets). * indicates $P < 0.05$.

(figure 4.7). The lower concentration of 1 nM cortisol did not significantly affect expression levels of the cytokines measured.

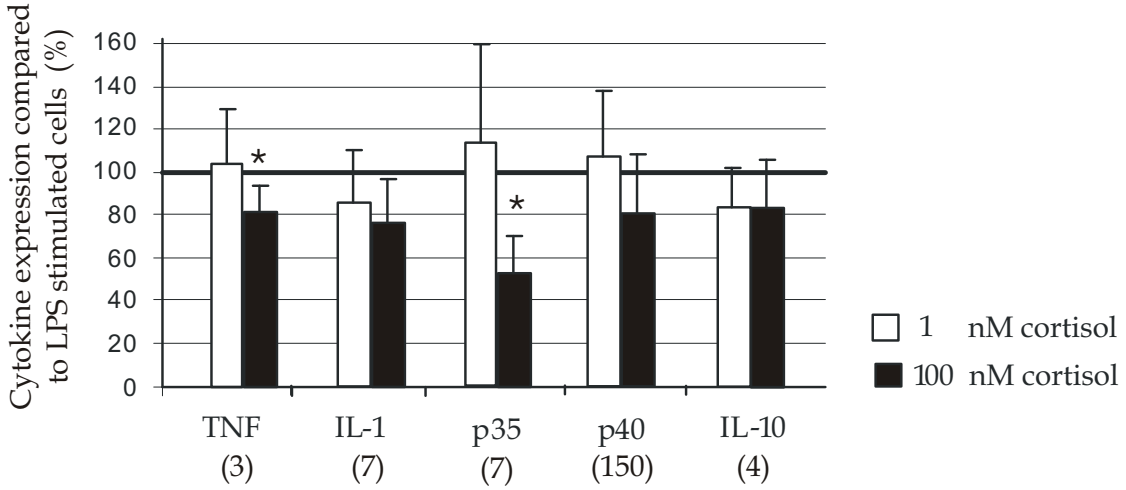


Figure 4.7: Cytokine mRNA expression in LPS-stimulated head kidney phagocytes. Freshly isolated head kidney phagocytes were stimulated for 4 hr with 30 µg/ml LPS or a combination of LPS with either 1 nM or 100 nM cortisol. Data of four control fish are shown as percentage of LPS-induced expression (set at 100 %, represented by the thick line). Average increase of mRNA expression after LPS treatment is shown in (brackets). * indicates $P < 0.05$.

4.4 Discussion

Common carp expresses four different corticoid receptors (GR1a, GR1b, GR2 and MR) and all four receptors are widely expressed in the central nervous system and pituitary gland (Stolte *et al.*, 2008a) as well as in peripheral organs. We here show differential roles for these glucocorticoid receptors (GRs) in cortisol-mediated immune modulation. We demonstrate constitutive expression of all cortiocosteroid receptors during development and in adult peripheral organs, their transactivation efficiency, and possible role in stress-related and immune modulation, as assessed by down-regulation of expression of phagocyte pro-inflammatory mediators.

MR mRNA expression in typical immune tissues such as head kidney, peripheral blood lymphocytes (PBL) and thymus is very low. To better appreciate the functional consequences of this low constitutive expression it is important to know the transactivation capacity, *i.e.* the concentration of the natural ligand (cortisol) required to activate or repress an effector gene. Recent experiments (Stolte *et al.*, 2008a) have shown that GR2 is the most sensitive receptor (EC_{50} 2.4 nM) whereas MR (EC_{50} 4.0 nM) and GR1 (EC_{50} 7.2 nM) are less sensitive. Considering the very low constitutive expression of the MR in immune organs and only moderate cortisol sensitivity we hypothesise that GRs rather than the MR are predominantly involved in transmitting stress signals to the immune system.

Based on the widespread corticoid receptor expression in adult immune organs and our demonstration that corticosteroid receptor levels in the whole embryo are comparable to levels in separate organs of adult fish as from 4 days post-fertilisation (dpf), it is tempting to hypothesise existence of cortisol-induced immune modulation at this early age. Alternatively, cortisol may play a role in the development of the immune system. Common carp show fast embryonic development of both the immune and the stress axis. After hatching at 2 dpf the larvae start feeding at 4 dpf when the yolk has been resorbed. Intriguingly, as early as 2 dpf endogenous ACTH and cortisol is produced and embryos show a 'stress response': whole body cortisol levels increase after handling (Sampath-Kumar *et al.*, 1997; Stouthart *et al.*, 1998; Flik *et al.*, 2002). Around the same time (2 dpf), the developing immune system is capable of responding to an immune stimulus; LPS increased IL-1β and *i*NOS expression (Huttenhuis *et al.*, 2006). Since both the immune system and the stress axis are present around hatching, we assume that early life cortisol-induced immune modulation can affect the developing immune system.

The occurrence of two splice variants of GR1 in carp reminds strongly of the situation in rainbow trout (*Oncorhynchus mykiss*) and Burton's mouthbrooder (*Haplochromis burtoni*),

and apparently such phenomenon is common in fishes (Greenwood *et al.*, 2003; Stolte *et al.*, 2006). Alternative splicing introduces 27 nucleotides in the DNA binding domain, which translate into a nine amino acid insert (WRARQNADG). Although this insert is conserved in evolutionary distantly related fish, rainbow trout and common carp are separated by 160 million years of evolution (Volff, 2005), other vertebrates do not show this insert (Stolte *et al.*, 2006). However, other inserts at the same location in the DNA binding domain have been reported in both GR and MR of different vertebrate species (Bloem *et al.*, 1995; Brandon *et al.*, 1991; Rivers *et al.*, 1999) and were shown to affect DNA binding and resulting transactivation capacity of the receptor (Wickert and Selbig, 2002). Human GR-γ (Ray *et al.*, 1996; Rivers *et al.*, 1999) and the cotton top marmoset (*Saguinus oedipus*) GR (Brandon *et al.*, 1991), show an extra Arginine (R), but its function was predicted to be comparable to wild type (Wickert and Selbig, 2002). Experiments, however, showed that although the transactivation capacity was unaffected, its V_{max} is diminished (Ray *et al.*, 1996). Our model of the three-dimensional structure of the splice variant, based on the rat GR DNA binding domain crystal structure, predicts that addition of nine amino acids extends the loop after the interfinger alpha helix. This loop protrudes outside of the protein, and may thus be expected not to interfere with receptor DNA-binding. Our results corroborate a prediction for the rainbow trout GR-insert (WRARQNTDG) that also showed a loop extending to the outside of the protein (Wickert and Selbig, 2002). As both predictions were based on the DNA binding domain only, it remains uncertain if this prediction would hold for the entire protein. Transactivation experiments confirmed that the nine amino acid insert did not affect the capacity to activate a luciferase gene under control of a MMTV promoter. The EC_{50}s are comparable for the variant with (GR1a) and without (GR1b) the insert and maximal activation was not altered. Also, EC_{50}-values for the potent synthetic steroid dexamethasone (GR1a 2.4 $\pm$ 3.8 nM and GR1b 2.9 $\pm$ 2.4 nM) were comparable as well (data not shown). Although these results show that alternative splicing does not affect DNA binding and resulting activation of transcription, the insert might affect interactions with co-activators and co-repressors (Kumar and Thompson, 2005). As the transactivation capacity for GR1a and GR1b are not significantly different, the mRNA expression levels of the variants combined can be summed and compared with GR2 expression levels. This results in similar relative expression levels in most immune related tissues, except for PBL that show an overrepresentation of GR2 mRNA expression. Recently we forwarded the hypothesis of a 'sensitive' GR2 receptor that can induce transcription at basal cortisol levels, and an 'insensitive' GR1 receptor that requires stress levels of cortisol to induce transcription (Stolte *et al.*, 2008a). Our current data are consistent with this hypothesis.

To investigate differential roles of CRs in immune cells we determined receptor expression profiles after either an immune stimulus and/or a stress stimulus in head kidney phagocytes. In isolated head kidney phagocytes constitutive GR mRNA expression is higher and constitutive MR mRNA expression lower than in whole head kidney, which further augments the predominance of GR over MR in immune cells. Glucocorticoid receptor levels of head kidney phagocytes were slightly increased by treatment with stress levels (100 nM) of cortisol. Earlier results showed downregulation of GR binding sites in peripheral blood leukocytes of carp fed with cortisol-containing food (Weyts *et al.*, 1998c), probably as a result of receptor translocation to the nucleus. In time, this might lead to increased mRNA levels to replenish GR numbers. Indeed, only after 24 hr the increase in GR expression was significant. LPS treatment however, quickly induced GR1 mRNA expression, whereas GR2 expression was inhibited. This might reflect a temporal surge of the 'stress' GR1 receptor expression to increase sensitivity for feedback control as was shown for murine macrophages (Salkowski and Vogel, 1992). After LPS treatment, pro-inflammatory cytokine expression levels (IL-1β, TNF-α, IL-12 (subunits p35 and p40) increase drastically with concomitant increase of nitric oxide (NO) (via inducible nitric oxide synthase) and toxic oxygen and nitrogen radicals directed to kill the invading pathogen (Engelsma *et al.*, 2003b; Huising *et al.*, 2006c; Saeij *et al.*, 2003b, 2000). This response is attenuated, likely to prevent detrimental and possible lethal effects. Indeed, in mice with GR-deficient macrophages higher mortality is seen after LPS treatment (Bhattacharyya *et al.*, 2007). Although fish do not suffer from septic shock, they do show a strong pro-inflammatory response with high production of oxygen and nitrogen radicals which requires a firm balance to ensure effective pathogen clearance and prevent damage to the host. In mammals GR activity mediates immune suppression through inhibition of transcription factors such as activator protein (AP-1) and nuclear factor kappa B (NFκB) that regulate expression of cytokines, inflammatory enzymes and inflammatory receptors (Rhen and Cidlowski, 2005). As intracellular pathways are much conserved throughout the vertebrate lineage, we predict the same inhibitory mechanisms for fish. Indeed NO production (under control of the inflammatory *i*NOS) was significantly decreased after treatment with a NFκB inhibitor (Saeij *et al.*, 2003a). LPS-induced expression of pro-inflammatory cytokine IL-1β could be blocked by NFκB inhibition (Engelsma *et al.*, 2003b).

To assess which CRs mediated immune modulation in common carp, we determined the ability of cortisol to affect LPS-induced cytokine production. Basal plasma cortisol levels in carp are below 20 ng/ml and can increase to well over 200 ng/ml during stress. Roughly 80 % of cortisol in circulation is bound to plasma proteins, which leaves a mere 20 % of the total

free and bioactive (Flik and Perry, 1989). With 1 nM (≈ 2 ng/ml in plasma) and 100 nM (≈ 200 ng/ml in plasma) cortisol in our cultures we could thus discriminate the cortisol concentration required to induce transcription by the GRs tested. At 1 nM cortisol, only the sensitive GR2 is activated, at 100 nM all three GRs will become maximally activated. The requirement of high levels of cortisol to induce inhibition of cytokine expression is in accordance with results of Saeij *et al.*, for IL-β, TNF-α and *i*NOS expression in head kidney phagocytes stimulated with lysate of the blood parasite *Trypanoplasma borreli* (Saeij *et al.*, 2003c). In head kidney phagocytes constitutive expression of GR1 (combined) and GR2 are similar, and these levels were not differentially regulated during our experiments. Despite the fact that high levels are necessary to initiate cytokine downregulation, not all immune modulation requires high levels of cortisol to take effect. Indeed, lymphocytes and especially B-lymphocytes, show high sensitivity for cortisol; significant inhibition of PBL proliferation and induction of apoptosis was measured at cortisol levels as low as 3.6 ng/ml, which can be due to activation of GR2 (Weyts *et al.*, 1997). The strong over-representation of GR2 over combined GR1 expression in PBL suggests a role for cortisol in immune functions in non-stressed fish as well.

In conclusion differential gene expression of the duplicate GRs or splice variants is found in endocrine as well as in immune organs and cell types. The immune modulatory response of these receptors appears physiologically important, they are not only steered by endocrine signaling as an immune stimulus strongly and differentially regulates their expression profiles in leukocytes.

Ackowledgements

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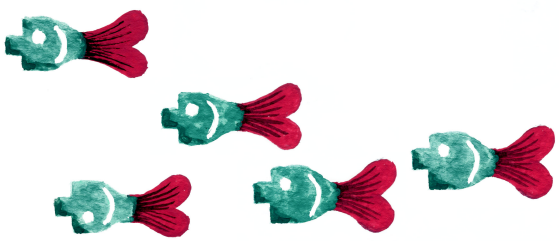

'Imagine a scientist being merely an animal . . . '

T.H. White, The book of Merlyn

5

The immune response differentially regulates Hsp70 and glucocorticoid receptor expression *in vitro* and *in vivo* in common carp (*Cyprinus carpio* L.)

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Abstract

Heat shock or stress proteins and glucocorticoids (cortisol) regulate a sequential pro-inflammatory and anti-inflammatory cytokine expression profile to effectively kill pathogens, whilst minimising damage to the host. Cortisol elicits its effects through the glucocorticoid receptor (GR) for which Hsp70 and Hsp90 are required as chaperones. In common carp, (*Cyprinus carpio*) duplicated genes and splice variants with different cortisol sensitivities exist. We investigated the expression profiles of heat shock proteins Hsp70, Hsc70, Hsp90α and Hsp90β and the three different variants of GR *in vitro* in and *in vivo* to define their role in immune modulation. A rapid transient induction of Hsp70 and GR1 (a and b) was seen after LPS treatment *in vitro* in head kidney phagocytes; cortisol-treatment does not affect constitutive or LPS-induced expression of Hsp70 or GR1 expression. *In vivo* zymosan-induced peritonitis upregulated GR and Hsp70 expression which appears to increase sensitivity for cortisol-induced immune modulation and correlates with inhibition of zymosan-induced expression of pro-inflammatory cytokines. Infection with the blood parasite *T. borreli* decreases GR1 expression in head kidney and thymus, but increases GR2 expression in spleen. This appears a result of increased numbers of B-lymphocytes, indicative of initiation of an adaptive immune response, with high constitutive GR2 expression. Differentially regulated expression of Hsp70 and of glucocorticoid receptor variants with different cortisol sensitivities, underlines their physiological importance in a balanced immune response.

5.1 Introduction

In mammals, regulation of the biphasic, pro-inflammatory and anti-inflammatory, immune response, is of critical importance and can be influenced by a glucocorticoid response and heat shock proteins. After infection a strong pro-inflammatory response is initiated by the immune system. An appropriate stress response will attenuate the release of pro-inflammatory cytokines, cytotoxic cell activity, and production of reactive oxygen species and stimulate release of anti-inflammatory cytokines and molecules to create a balanced immune response and by doing so, effectively protects against the pathogen and simultaneously reduces damage to the host (Elenkov and Chrousos, 2006). This glucocorticoid response is critical for host survival, as demonstrated by the fact that impaired HPA axis activity (adrenalectomy or hypophysectomy) or glucocorticoid receptor (GR) blockade enhances the severity of the infection (Webster and Sternberg, 2004). The hypothalamo-pituitary-adrenal (HPA) or stress axis is activated during various pathogen infections, resulting in increased circulating glucocorticoid (cortisol or corticosterone) levels. Cortisol passes through cell membranes and binds to intracellular transcription factors, such as the glucocorticoid receptor (GR). In binding, the heat shock proteins Hsp70 and Hsp90 are essential as chaperones (Pratt and Toft, 2003). The hormone receptor complex formed will translocate into the nucleus where it activates or represses transcription of effector genes. Additionally, GR can interact with other transcription factors such as activator protein (AP-1) and nuclear factor kappa B (NFκB) that regulate expression of cytokines, inflammatory enzymes and receptors for inflammatory molecules (Rhen and Cidlowski, 2005).

Besides the organismal stress response, a cellular stress response exists, which is mediated by various heat shock (or stress) proteins (Hsp's) classified into families based on their molecular weight. Although Hsp's initially received their name after discovery in temperature shocked *Drosphila busckii* (Ritossa, 1962), these proteins are induced in response to a variety of stressors, particularly in response to those stressors that tend to denature proteins. However, Hsp's are also constitutively expressed and critical for protein metabolism. Hsp90 shows abundant constitutive expression and has a role in maintaining the cytoskeleton, but is only mildly regulated after stress (Young *et al.*, 2003). In contrast, Hsp70, which assists in folding of nascent polypeptides and acts as a molecular chaperone shows low constitutive expression, but is strongly induced after stress (Liberek *et al.*, 2008). Interestingly, also a constitutive member of the Hsp70 family exists; Hsc70, which has a role in clathrin-mediated endocytosis (Eisenberg and Greene, 2007) and is hardly induced after stress (Suganuma *et al.*, 2002).

Hsp70 also serves an important role in the immune response. Comparable to the glucocorticoid response, induction of the heat shock response (Hsp70) suppresses LPS-induced production of pro-inflammatory cytokines through modulation of the NF-κB cascade (Shi *et al.*, 2006) and the JNK/AP-1 signaling pathway (Chen *et al.*, 2005; Wang *et al.*, 2003) and augments production of anti-inflammatory cytokines (Wang *et al.*, 2001). On the other hand, Hsp70 is released extracellularly, through physiological secretion mechanisms or after necrosis, and acts as a danger signal (Calderwood *et al.*, 2007). Recognition of this extracellular Hsp70 by Toll-like receptor 4 (TLR4) has been widely described and results in release of pro-inflammatory cytokines, through NFκB and/or MyD88 pathways (Chase *et al.*, 2007; Doz *et al.*, 2008; Satoh *et al.*, 2006). Moreover, Hsp70 released into the extracellular environment in a membrane-associated form activates macrophages and natural killer cells (Elsner *et al.*, 2007; Vega *et al.*, 2008).

Teleostean fishes produce stress signals via their hypothalamo-pituitary-interrenal (HPI) axis that is analogous to the mammalian HPA axis. Also formation of the cortisol-receptor complex and transactivation or transrepression is considered to utilise intracellular routes similar to those described in mammals (Stolte *et al.*, 2006). Indeed, for rainbow trout (*Onchorhynchus mykiss*) Hsp70 was demonstrated in the GR receptor complex and stress (heat stress and cortisol-treatment) was found to stimulate this association of Hsp70 with the glucocorticoid receptor (Basu *et al.*, 2003). Teleostean fishes, in contrast to mammals, have three different glucocorticoid receptors as a result of gene duplication (GR1 and GR2) and alternative splicing (GR1a and GR1b) (Stolte *et al.*, 2006). The receptors require basal (GR2) or stress (GR1) levels of cortisol to induce transcription of effector genes (Stolte *et al.*, 2008a). Therefore, stress-induced immune modulation can be regulated by three different receptors, alone or in conjunction.

We investigated the regulation of mRNA expression profiles of heat shock proteins and three different glucocorticoid receptors as an indication for their role in immune modulation in carp, a representative of the earliest vertebrates with both innate and adaptive arms of the immune system. We show that an immune stimulus modulates expression of Hsp70 and the insensitive ('stress') GR1 *in vitro* and *in vivo*, whereas *in vitro* cortisol-treatment does not affect Hsp or GR expression in leukocytes. The differential expression profiles of heat shock proteins and glucocorticoid receptors therefore, appear of physiological importance to mediate a balanced immune response after pathogen exposure.

5.2 Experimental procedures

5.2.1 Animals

Nine month old carp (150–200 g, *Cyprinus carpio* L.), were kept at 23 °C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7 % of their estimated body weight. The cross 'R3×R8' is the offspring of Hungarian (R8) and Polish (R3) strains (Irnazarow, 1995). Experiments were repeated with fish reared from different batches of eggs. The zymosan experiment was carried out with young fish (50–60 g), from the Department of Immunology, Polish Academy of Science, Golysz, Poland (R7xW) reared at 20 °C in recirculating tap water. This experiment was conducted according to license no. 16/OP/2001 from the Local Ethical Committee. All other experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

5.2.2 Tissue preparation

Fish were anaesthetised with 0.2 g/l tricaine methane sulfonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l $NaHCO_3$ (Merck, Darmstadt, F.R. Germany). Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe with a 21 gauge needle. Next, fish were killed by spinal transection and organs and tissues were carefully removed, snap-frozen in solid CO_2 or liquid N_2, and stored at -80 °C until RNA isolation.

5.2.3 *In vivo* experiments

Zymosan-induced peritonitis. A sterile peritonitis was induced with zymosan (Chadzinska *et al.*, 2008). Animals were either untreated (intact fish), sham injected, or i.p.-injected with freshly prepared zymosan A (2 mg/ml, 1 ml/50 g body weight; Sigma-Aldrich, Zwijndrecht, The Netherlands). At selected time points animals were sacrificed and their peritoneal cavities lavaged with 1 ml of ice cold phosphate-buffered saline (PBS).

Blood parasite infection. Fish were infected with *Trypanoplasma borreli* (cloned as described by Steinhagen *et al.* (1989), by intraperitoneal injection of 1×10^4 parasites per fish in 100 µl RPMI (n=4), or fish were injected with 100 µl RPMI (n=4; controls). Parasitemia was measured using a Bürker counting chamber.

5.2.4 *In vitro* experiments

Head kidney phagocytes were obtained by passing head kidney tissue through a 100 µm nylon mesh (BD Bioscience, Breda, The Netherlands) with carp RPMI (cRPMI; RPMI 1640, Invitrogen Carlsbad, CA, USA; adjusted to carp osmolality (280 mOsm/kg) and washed twice. The cell suspension was layered on a discontinuous Percoll (Sigma-Aldrich) gradient (1.020, 1.060, 1.070 and 1.083 g/cm^3 interphase) and centrifuged 25 min at 800 *g* with the brake disengaged. Cells at the 1.070 and 1.083 g/cm^3 were collected and washed twice with cRPMI and once with cRPMI^{++} *i.e.* cRPMI supplemented with 0.5 % pooled carp serum, 1 % glutamine (Cambrex, Verviers, Belgium), 1 % penicillin G (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1 % streptomycin sulphate (Sigma-Aldrich). Cell populations thus obtained were found to be similar as described before (van Kemenade *et al.*, 1994); at 1.070 g/cm^3, predominantly macrophages (65 %), with 10 % granulocytes and 25 % small macrophages and lymphocytes are obtained and at 1.083 g/cm^3, predominantly neutrophilic granulocytes (85 %) with 15 % macrophages are found. Stimulations were carried out in in duplicate in cRPMI^{++} at 5×10^6 cells per well (in 500 µl) in a 24 well cell culture plate. Cells were stimulated for 2-48 hr at 27 °C at 5 % CO_2 with 30 µg/ml LPS (Lipopolysaccharide from *Escherichia coli 055:B5*, Sigma-Aldrich), or with 100 nM cortisol (Sigma-Aldrich) or a combination of LPS and cortisol. Control cells received medium only and experiments were repeated for four independent fish. After stimulation supernatant was removed and cells were collected in 300 µl RLT buffer from the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands (duplicate treatments were pooled) and stored at -80 °C.

5.2.5 RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen, Carlsbad, CA, USA), as suggested by the manufacturer. Total RNA was precipitated in isopropanol, washed with 75 % ethanol and dissolved in nuclease-free water. RNA of cells was isolated as described by the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) strictly according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry and integrity was confirmed by electrophoresis on a 1.5 % agarose gel before proceeding with cDNA synthesis.

5.2.6 DNAse treatment and first strand cDNA synthesis

For each sample a '–RT' (non reverse transcriptase) control was included. One microliter of 10× DNase-I reaction buffer and 1 µl DNase-I (Invitrogen, 18068-015) was added to

1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65 °C for 10 min. To each sample, 300 ng random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5× First Strand buffer (Invitrogen), 2 µl 0.1 M dithiothreitol (DTT) and 40 Units RNAse Out (Invitrogen 10777-019) were added and the mix was incubated for 10 min at room temperature and for and additional 2 min at 37 °C. To each sample (not to the '–RT' controls) 200 U Superscript-II RNase H^- Reverse Transcriptase (RT; Invitrogen, 18064-014) was added and reactions were incubated for 50 min at 37 °C. Demineralised water was added to a final volume of 100 µl and samples were stored at -20 °C until further use.

Table 5.1: Primers used for gene expression studies. Table shows sequences and GenBank accession numbers.

Gene	FW primers	RV primers	Acc. No.
GR1a	GTT-CGT-CGG-ATC-AGC-AAG-C	CTG-CGT-TTT-GTC-GTG-CTC-TC	AJ879149
GR1b	GTT-CGT-CGG-ATC-AGC-AAG-C	TTG-TGC-TGC-CCC-TCT-ACG	AM697886
GR2	GGA-GAA-CAA-CGG-TGG-GAC-TAA-AT	GGC-TGG-TCC-CGA-TTA-GGA-A	AM183668
Hsp70	GGC-AGA-AGG-TGA-CAA-ATG-CA	TGG-GCT-CGT-TGA-TGT-TCT-CA	AY120894
Hsc70	CCA-GAC-TGC-AGA-AAA-GGA-TGA-GTA	CCG-CCC-TTC-CAG-GAA-ATC	AY120893
Hsp90α	TTA-CCC-CAT-TAC-GCT-CTT-CGT-T	CAA-TCT-TGG-GTT-TGT-CTT-TCT-CAT-C	AF170295
Hsp90β	GGC-TAC-CCA-ATC-ACT-CTC-TTC-GT	GGG-TTT-GTC-TTC-GCC-TTC-CT	AF170296
40S	CCG-TGG-GTG-ACA-TCG-TTA-CA	TCA-GGA-CAT-TGA-ACC-TCA-CTG-TCT	AB012087

5.2.7 Real-time quantitative PCR

PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and *PRIMER3* software (Rozen and Skaletsky, 2000)was used to design primers for use in real-time quantitative PCR (RQ-PCR) (table 5.1). For RQ-PCR 5 µl cDNA and forward and reverse primers (300 nM each) were added to 7 µl Brilliant® SYBR® QPCR Master Mix (Stratagene, La Jolla, CA, USA) and demineralised water was added to a final volume of 14 µl. RQ-PCR (10 min 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C followed by 1 min at 60 °C) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed with comparative quantitation of the Rotor-gene Analysis Software V5.0. Constitutive gene expression in organs and tissues was determined as a ratio of target gene *vs* reference gene and was calculated according to the following equation.

$$\text{ratio} = \frac{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}}}{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}}} \tag{5.1}$$

where E is the amplification efficiency and Ct is the number of PCR-cycles needed for the signal to exceed a predetermined threshold value. Expression following *in vitro* or *in vivo* stimulation was determined relative to the expression of non-stimulated cells or control fish according to the following equation (Pfaffl, 2001).

$$\text{ratio} = \frac{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}(\text{control} - \text{sample})}}{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}(\text{control} - \text{sample})}} \tag{5.2}$$

Dual internal reference genes (40S ribosomal protein and β-actin) were incorporated in all RQ-PCR experiments; results were similar following standardisation to either gene. Non-template controls were included for each gene in each run and '–RT'-controls were included in all experiments; no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and melting curve profiles. The product of each template was checked at least once by sequencing.

5.2.8 Statistics

Statistic analysis was performed using SPSS 12.0.1 software. Differences in corticosteroid receptor and heat shock protein expression were evaluated with a Student's *t*-test and $P < 0.05$ was accepted as significant. Homogeneity was tested with Levene's test and we corrected the Student's *t*-test for unequal variances when necessary. Significance of differences after zymosan-induced peritonitis was determined using ANOVA with post hoc Tukey's test. Data are represented as average and error bars indicate standard deviation * indicates $P < 0.05$, ** indicates $P < 0.01$.

5.3 Results

Constitutive heat shock protein expression in immune organs and cells. Hsp70, Hsc70, Hsp90α and Hsp90β are constitutively expressed in all immune organs and cell types tested (figure 5.1). Hsp90β showed very high constitutive expression in all organs examined, 500-fold higher than Hsp70 and Hsc70 expression levels. Hsp90α expression levels are very low, ten-fold lower than Hsp70 and Hsc70. Hsp70 expression is very low in head kidney; 10-fold to 20-fold lower than in other immune organs. Hsc70 and Hsp90α expressions in peripheral blood lymphocytes (PBL) are very low. Heart shows 10-fold, 30-fold and 500-fold higher expression for Hsp70, Hsc70 and Hsp90α, respectively compared to other organs (data not shown). In ovaries Hsp70 expression is 15-fold higher than in other organs (data not shown).

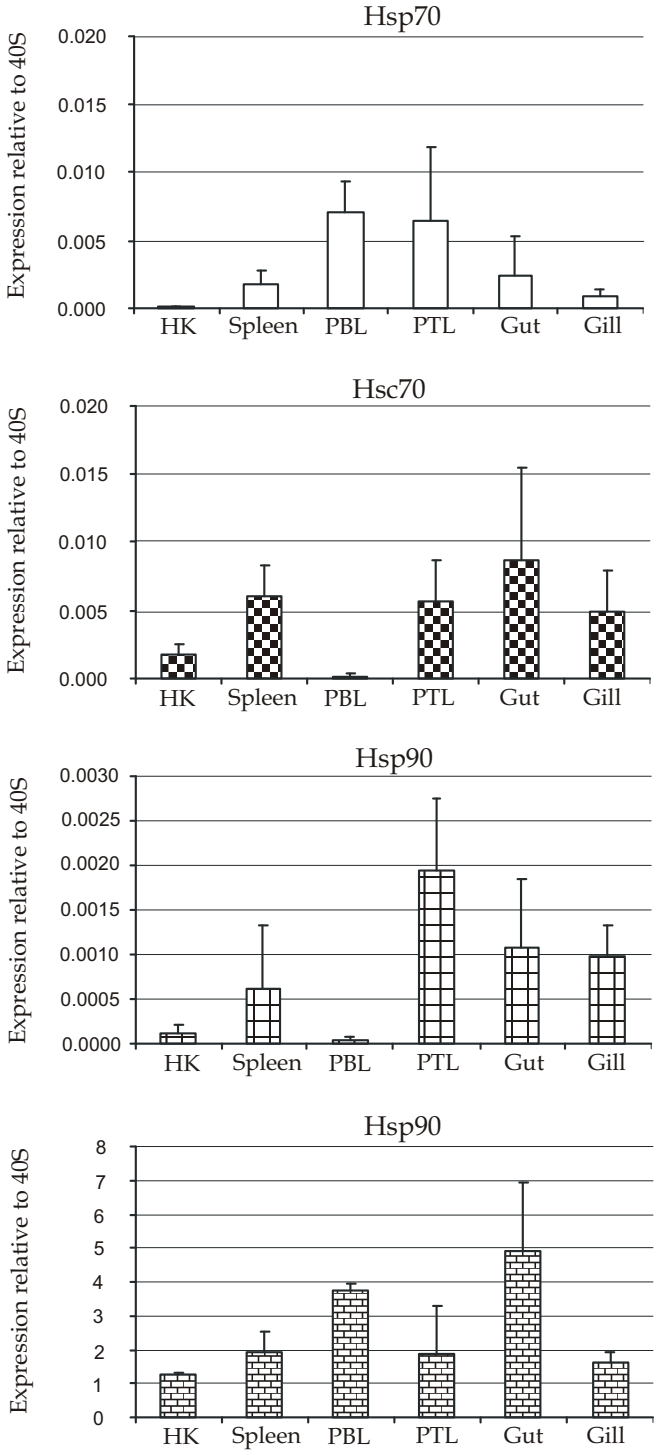


Figure 5.1: Constitutive heat shock protein mRNA expression in peripheral organs. cDNA of different organs, or freshly isolated blood or peritoneal leukocytes, was used as template for quantitative real time PCR. Messenger RNA expression data of four control fish is shown relative to the housekeeping gene 40S. HK; head kidney, PBL; peripheral blood leukocytes, PTL, peritoneal leukocytes. Constitutive GR expression in head kidney; GR1a 0.075 $\pm$ 0.008, GR1b; 0.045 $\pm$ 0.003, GR2; 0.156 $\pm$ 0.013. * indicates $P < 0.05$.

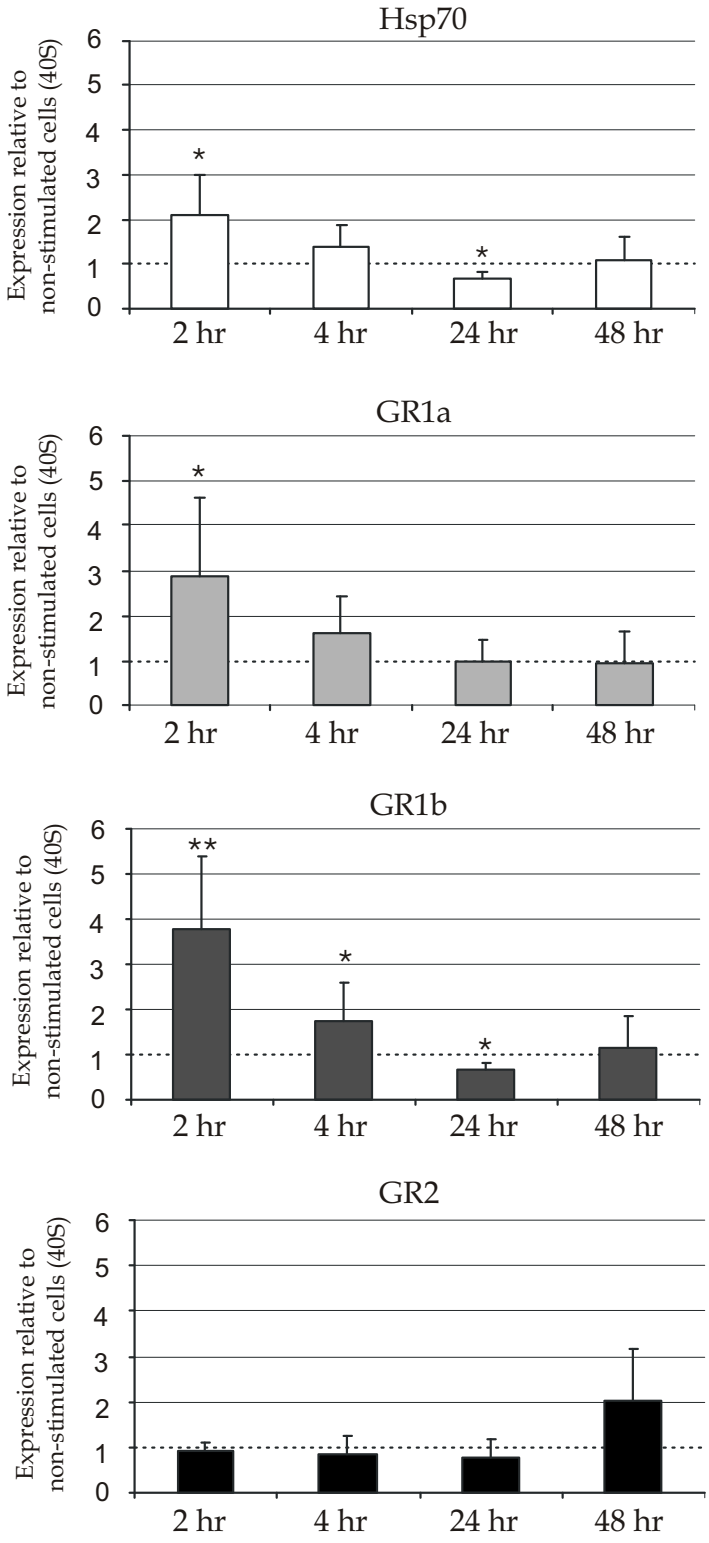


Figure 5.2: Glucocorticoid receptor and heat shock protein 70 mRNA expression in head kidney phagocytes after *in vitro* stimulation with 30 µg/ml LPS. cDNA of four (24 and 48 hr) or six to eight (2 and 4 hr) individual stimulation experiments was used as template for quantitative real time PCR. Messenger RNA expression data is shown as ×-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S. * indicates $P < 0.05$.

LPS-stimulation rapidly and transiently increases GR1 and Hsp70 expression. Hsp70 mRNA expression was significantly increased in head kidney phagocytes after 2 hr stimulation with 30 μg/ml LPS (figure 5.2). After 24 hr Hsp70 expression was significantly decreased compared to non-stimulated control cells. LPS-stimulation did not differentially regulate expression profiles of any of the other heat shock proteins investigated (table 5.2). GR1a and GR1b expression showed a significant increase early after stimulation (GR1a; 2 hr, GR1b 2 and 4 hr), which decreased over time. After 24 hr GR1b expression was decreased compared to non-stimulated phagocytes and after 48 hr no difference could be detected between stimulated and non-stimulated cells. GR2 mRNA did not significantly change at any of the time points investigated. After 48 hr no difference could be detected between stimulated and non-stimulated cells for any of the genes tested.

Table 5.2: Heat shock protein mRNA expression in head kidney phagocytes after *in vitro* treatment with 30 μg/ml LPS. cDNA of four (24 and 48 hr) or six to eight (2 and 4 hr) individual stimulation experiments was used as template for quantitative real time PCR. Messenger RNA expression data is shown as ×-fold increase compared to non-stimulated control cells (set at 1), standardised for the housekeeping gene 40S. * represents $P < 0.05$.

Gene	2 hr		4 hr		24 hr		48 hr	
	avg.	SD	avg.	SD	avg.	SD	avg.	SD
Hsp70	2.12 *	0.88	1.36	0.51	0.69 *	0.16	1.09	0.54
Hsc70	1.55	0.93	1.04	0.42	0.80	0.35	1.66	0.79
Hsp90α	3.19	3.27	1.75	0.98	0.95	0.63	1.29	0.59
Hsp90β	1.35	0.44	1.41	0.80	2.11	2.77	2.39	1.89

In a separate experiment we investigated whether addition of 100 nM of cortisol would affect LPS-induced GR and Hsp70 expression in head kidney phagocytes after 2 and 4 hr (table 5.3). Again LPS consistently increased Hsp70 and GR1 (a and b) expression. Cortisol treatment did not differentially regulate Hsp70 and GR expression. When LPS and cortisol were combined, expression profiles did not differ from cells treated with either LPS or cortisol alone. Cortisol did not differentially regulate expression of Hsp70, Hsc70, Hsp90α and Hsp90β. Addition of cortisol to LPS-treated cells did not change heat shock mRNA expression.

Zymosan-induced peritonitis increases GR and Hsp70 expression. GR and Hsp70 mRNA expression levels in peritoneal leukocytes and head kidney were determined during the first week of inflammation after zymosan injection. In peritoneal leukocytes (figure 5.3a), GR1 (a and b) expression was increased from 24 hr to 48 hr and showed a second peak after a 16 hr. GR2 expression did not significantly change at any time point in peritoneal leuko-

Table 5.3: Glucocorticoid receptor and heat shock protein 70 mRNA expression in head kidney phagocytes after *in vitro* treatment with 30 µg/ml LPS, 100 nM of cortisol or a combination of both. cDNA of four individual experiments was used as template for quantitative real time PCR. Messenger RNA expression after 2 (a) or 4 (b) hr is shown as ×-fold increase compared to non-stimulated control cells (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S. * represents $P < 0.05$.

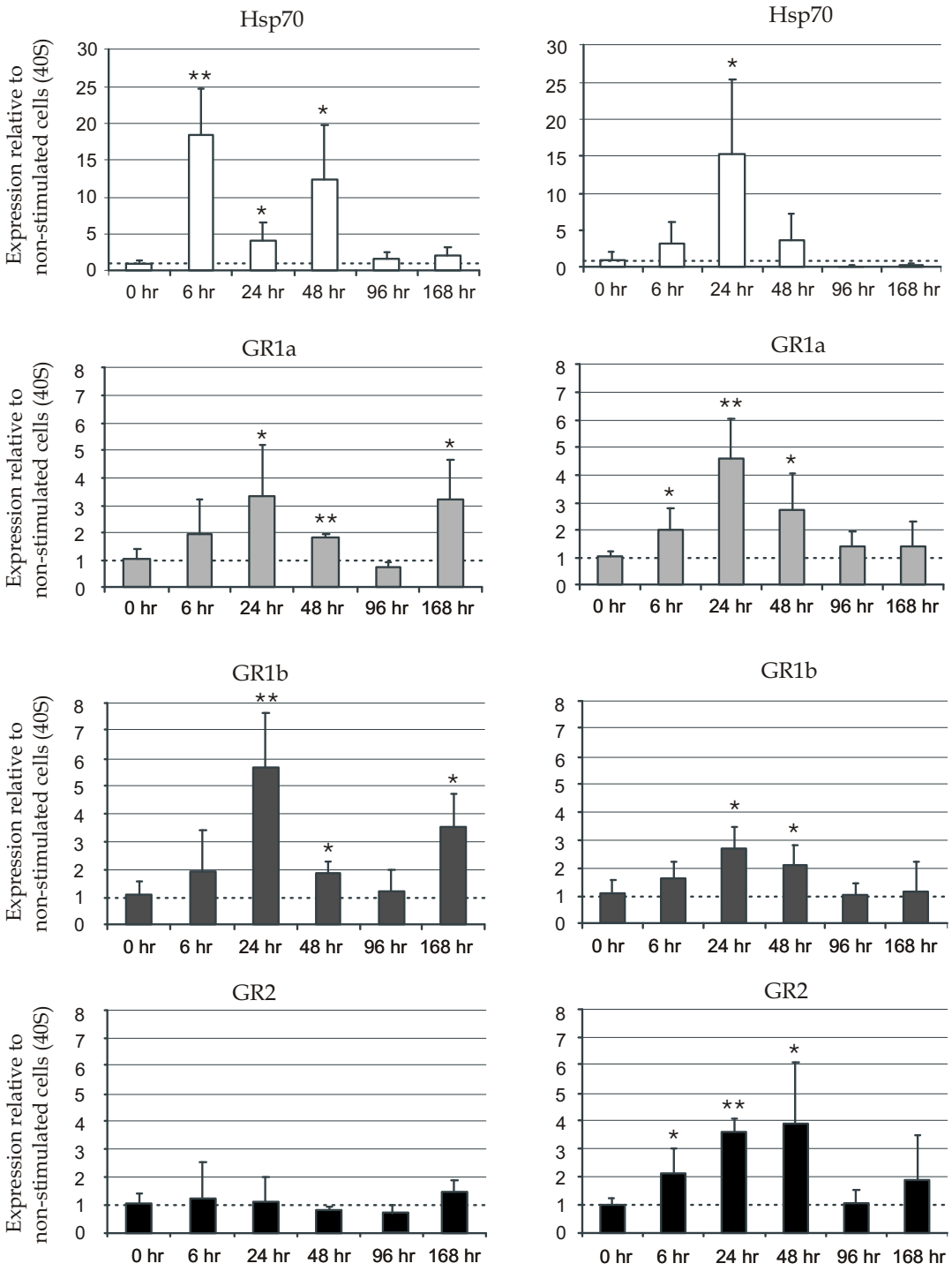
(a) mRNA expression after 2 hr

Gene	Cortisol 2 hr		LPS 2 hr		LPS & Cortisol 2 hr	
	avg.	SD	avg.	SD	avg.	SD
GR1a	1.42	0.60	2.75	2.10	2.71	2.44
GR1b	1.52	0.84	3.14	2.05	2.63	0.96
GR2	0.83	0.14	0.90	0.26	0.98	0.27
Hsp70	2.39	1.16	2.37 *	0.83	1.61	1.01
Hsc70	1.12	0.31	1.88	1.16	1.49	0.95
Hsp90α	1.88	0.09	3.93	3.76	3.88	5.17
Hsp90β	1.23	0.49	1.22	0.50	1.44	0.69

(b) mRNA expression after 4 hr

Gene	Cortisol 4 hr		LPS 4 hr		LPS & Cortisol 4 hr	
	avg.	SD	avg.	SD	avg.	SD
GR1a	0.93	0.26	1.89	0.94	1.52	1.25
GR1b	1.00	0.21	1.93 *	0.55	1.45	0.77
GR2	1.19	0.29	1.02	0.39	1.06	0.24
Hsp70	1.91	1.59	1.42	0.76	2.20	2.15
Hsc70	1.60	1.20	0.76	0.39	1.87	2.40
Hsp90α	3.06	1.83	3.38	4.25	8.51	10.58
Hsp90β	1.36	0.57	0.96	0.23	1.55	0.90

cytes. Hsp70 mRNA expression was increased from 6 to 48 hr. Hsc70 expression decreased at 24 hr, and Hsp90α was increased at 6 hr but showed high individual variation. The expression profiles of Hsp90β did not significantly change (table 5.4a). In head kidney (figure 5.3b) GR1a was increased from 6 to 48 hr and GR1b was increased from 24 to 48 hr. GR2 expression was increased from 6 to 48 hr. Hsp70 mRNA expression showed a peak after 24 hr. Messenger RNA expression of Hsc70 was increased at 24 hr and Hsp90α expression was increased at 6 and 48 hr. Hsp90β expression was increased from 6 to 48 hr and was again increased at 168 hr (table 5.4b).



(a) Peritoneal leukocytes (b) Head kidney

Figure 5.3: Glucocorticoid receptor and heat shock protein 70 mRNA expression in peritoneal leukocytes (a) or head kidney (b) during zymosan-induced peritonitis. cDNA of four individual fish was used as template for quantitative real time PCR. Messenger RNA expression is shown as ×-fold increase compared to intact animals (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S. * indicates $P < 0.05$.

Table 5.4: Heat shock protein mRNA expression in peritoneal leukocytes (a) or head kidney (b) during zymosan-induced peritonitis. cDNA of four control and four injected fish was used as template for quantitative real time PCR. Messenger RNA expression is shown as ×-fold increase compared to intact animals (set at 1), standardised for the housekeeping gene 40S. * represents $P < 0.05$ and ** represents $P < 0.01$.

(a) Peritoneal leukocytes

Gene	0 hr		6 hr		24 hr		48 hr		96 hr		168 hr	
	avg.	SD	avg.	SD	avg.	SD	avg.	SD	avg.	SD	avg.	SD
Hsp70	1.00	0.40	18.5 **	6.18	4.03 *	2.50	12.34 *	7.39	1.71	0.93	2.20	1.04
Hsc70	1.00	0.35	1.21	0.80	0.84	0.52	0.48 *	0.05	0.65	0.70	1.10	0.39
Hsp90α	1.00	0.68	4.26	2.91	0.39	0.33	1.00	0.60	0.96	1.00	0.70	0.58
Hsp90β	1.00	0.72	1.76	0.61	0.90	0.22	1.64	0.80	0.70	0.04	1.47	0.65

(b) Head kidney

Gene	0 hr		6 hr		24 hr		48 hr		96 hr		168 hr	
	avg.	SD	avg.	SD	avg.	SD	avg.	SD	avg.	SD	avg.	SD
Hsp70	1.00	1.10	3.20	3.00	15.3 *	10.1	3.66	3.42	0.18	0.07	0.30	0.34
Hsc70	1.00	1.26	0.77	0.38	3.20 *	1.27	2.69	2.20	0.56	0.53	0.30	0.20
Hsp90α	1.00	0.62	3.50 *	1.68	28.4	34.2	6.08 *	1.74	2.70	3.80	3.90	3.50
Hsp90β	1.00	0.28	1.82 **	0.24	4.60 **	2.29	4.22 **	1.30	0.76	0.17	1.49 *	0.02

Differential GR expression after parasite infection. We determined head kidney GRs and Hsps mRNA expression levels at peak parasitemia (3 weeks post-infection, data not shown) after infection with *T. borreli* (figure 5.4). Hsp70 mRNA expression appeared to be increased in head kidney of infected fish, but was differentially regulated in thymus and spleen. After parasite infection, GR1a mRNA expression was consistently lower in head kidney and thymus and was unaffected in spleen. GR1b expression appeared decreased in head kidney. GR2 expression was not regulated by parasite infection in head kidney and thymus but was significantly increased in spleen. After parasite infection, Hsp90β was significantly increased in head kidney and spleen but not in thymus. Hsc70 and Hsp90α levels did not significantly change after parasite infection in any of the organs tested (data not shown)

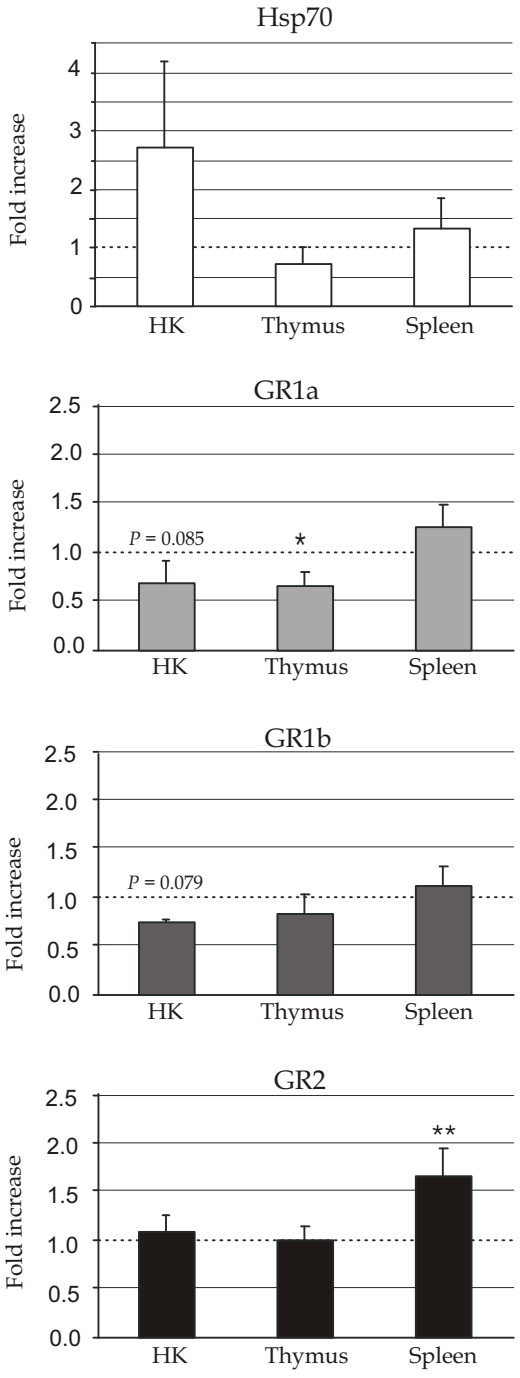


Figure 5.4: Glucocorticoid receptor and heat shock protein 70 mRNA expression in head kidney, thymus, and spleen during infection with blood parasite *T. borreli*. Three weeks after parasite infection, head kidneys of four control and four infected fish were used as template for real time quantitative PCR. Messenger RNA expression is shown as ×-fold increase compared to non-infected fish (set at 1, represented by the dotted line), standardised for the housekeeping gene 40S. * indicates $P < 0.05$.

5.4 Discussion

Extensive expression profiling after different *in vitro* and *in vivo* immune stimuli reveals that LPS-stimulation, inflammation or parasite infection, but not cortisol-treatment, differentially regulate heat shock protein and glucocorticoid receptor expression in immune tissues. This differential regulation appears to modify the sensitivity for glucocorticoid regulation and thereby regulate cytokine expression in immune cells.

Munck and coworkers (Munck *et al.*, 1984) proposed that 'Stress-induced increases in cortisol levels protect the organism against the normal reactions that are activated by the stress, rather than by the stress itself'. This notion rationalised the apparent paradox that a pathogen induces a stress response although glucocorticoids at moderate to high levels have anti-inflammatory properties. Glucocorticoids take part in creating a balance between pro-inflammatory and anti-inflammatory responses to effectively kill the pathogen, whilst minimising the damage to the host. Heat shock proteins can fulfil a similar role in generating a balanced cytokine expression profile and are increased after exposure to various stressors, both abiotic and biotic (pathogenic).

At early time points, *in vitro* stimulation of head kidney phagocytes with LPS transiently induces expression of the 'insensitive' or stress receptor GR1 (a and b), which is only activated at stress levels of cortisol, (Bury *et al.*, 2003; Stolte *et al.*, 2008b). Head kidney phagocytes thus appear to increase their sensitivity for cortisol, by up-regulation of GR expression, thereby facilitating cortisol-induced immune control early in the response. A similar result was found in murine macrophages, that upon stimulation with LPS transiently (from 2 to 16 hr) increased GR expression (Barish *et al.*, 2005). Moreover, to protect against endotoxin lethality, glucocorticoids have to be administered prior to, or in the early stages of the endotoxin challenge (Webster and Sternberg (2004) and refs therein). It is tempting to suggest that such a mechanism evolved already in fishes and has proven to be beneficial throughout the evolution of vertebrates.

Hsp70 expression is increased under conditions similar to those that increase GR1 expression, most likely to enhance cortisol sensitivity in immune cells. Hsp70 is required for ligand binding to GR and is constitutively low expressed compared to GR mRNA expression levels. Therefore, if this signal is strong and persistent, Hsp70 could be a limiting factor to convey the stress signal. Moreover, Hsp70 was shown to inhibit LPS-induced pro-inflammatory cytokine expression in mammals (Shi *et al.*, 2006). We hypothesise that a similar function exists in teleostean fishes. Indeed, expression of Hsp70 in head kidney phagocytes is rapidly and transiently induced by LPS treatment *in vitro*. We even observed

a slight down-regulation of Hsp70 expression, probably as a result of overcompensation (*e.g.* momentary strong decrease in expression to compensate for prior increase), before returning to normal levels. Similarly, in rainbow trout (*Oncorhychus mykiss*) Hsp70 expression slightly decreased after 12 hr of LPS stimulation (MacKenzie *et al.*, 2006).

Our hypothesis of a rapid and transient increase in Hsp70 and/or GR expression in response to immune stimulation was then addressed *in vivo*, by injection of the fish with the immune stimulant zymosan, a yeast cell wall component. The sterile peritonitis thus induced, increased both GR1 (a and b) and Hsp70 mRNA expression in peritoneal leukocytes (PTL), which can result from a migration of cells with high GR1 or Hsp70 expression into the inflamed tissue, and/or differentially regulated expression in the resident cells. After zymosan injection there is a strong influx of phagocytes, that reaches a maximum at 24 hr (Chadzinska *et al.*, 2008). In agreement with our hypothesis of a regulatory function of Hsp70 and GR during an immune response, the zymosan-induced increase in expression of pro-inflammatory cytokines interleukin-12 (IL-12) and IL-1β in PTL was attenuated from 48 hr onward. This follows the peak of GR1 expression at 24 and 48 hr and the increased Hsp70 expression from 6 to 48 hr. Moreover, expression of anti-inflammatory cytokine interleukin 10 (IL-10) in PTL is significantly increased from 48 hr onward (Chadzinska *et al.*, 2008). Interestingly, in head kidney during peritonitis, both GR1 and GR2 were upregulated at 24 and 48 hr, which possibly reflects a response of immune and/or endocrine (chromaffin and interrenal) cells, as in teleostean fishes the head kidney combines immune and endocrine functions (Gallo and Civinini, 2003). Experiments in gilthead seabream (*Sparus aurata)* showed induced GR expression after *in vivo* LPS treatment (Acerete *et al.*, 2007). The authors concluded that the increase in GR expression could be a result of increased cortisol release as a result of pro-inflammatory cytokine expression, which stimulated the HPI axis. We cannot exclude that the same mechanism takes place in carp as increased levels of corticosterone and cortisol were observed after intraperitoneal zymosan injection in mice (Scislowska-Czarnecka *et al.*, 2004) and in goldfish (unpublished observations), respectively. However, in the present experiments cortisol-treatment *in vitro* does not affect glucocorticoid receptor expression. Moreover, we found that although stress slightly increases GR expression in whole head kidney, cortisol does not enhance the sensitivity of immune cells (Stolte *et al.*, 2008b). Therefore an immune stimulus rather than increased cortisol levels appears to induce GR expression. Similarly, Hsp70 appears primarily regulated by an immune stimulus as cortisol-treatment *in vitro* did not affect Hsp70 expression. Furthermore, *in vivo* experiments showed that restraint stress did not elicit Hsp70 induction, (unpublished observations). In rainbow trout and Mozambique tilapia (*Oreochromis*

mossambicus) it was shown that cortisol administration did not affect Hsp70 levels (Basu *et al.*, 2001). Moreover, in separate experiments it was shown that handling stress (once or repetitive over days) and anaesthesia with TMS did not increase Hsp70 levels (Hosoya *et al.*, 2007; Vijayan *et al.*, 1997; Washburn *et al.*, 2002).

Injection with zymosan significantly induced both variants of Hsp90 expression in head kidney. Hsp90 is required for binding of cortisol to the GR, but it is not clear whether it is Hsp90α or Hsp90β, that is involved. As constitutive Hsp90α expression is very low, the strong but variable induction of expression after zymosan injection might reflect an increase in demand to facilitate binding to the GR. In rainbow trout increased Hsp90 levels were correlated with increased GR expression (Vijayan *et al.*, 2003). Combining of GR, Hsp70 and Hsp90 data suggests that regulated expression of GR and Hsp70 in leukocytes is primarily induced by immune stimuli and not necessarily by stress signaling.

As the early time points during zymosan-induced peritonitis mainly reflect an innate phagocyte-mediated response, we proceeded to investigate GR and Hsp expression after a parasite infection which evokes an adaptive immune response after three weeks (Joerink *et al.*, 2006; Saeij *et al.*, 2003a). The decreased GR1 expression in head kidney and thymus observed during parasite infection suggests an reduced cortisol sensitivity for the resident phagocytes and T-lymphocytes in those organs. Interestingly, in spleen, the 'sensitive' GR2, which is already activated at basal cortisol levels, is significantly increased. This can be explained by the large number of activated lymphocytes, to assist in the adaptive response. Indeed, greatly enlarged B-lymphocyte areas in spleen and a significant increase in serum antibody levels were detected after three weeks of infection (Forlenza *et al.*, 2008b). A differential response to cortisol-treatment has been shown before. B-lymphocytes, which show high expression levels of GR2 compared to other immune cells (Stolte *et al.*, 2008a), are very sensitive to cortisol and only require very low levels enter into apoptosis (Weyts *et al.*, 1998a). Whereas T-cells are less sensitive and neutrophilic granulocytes are actually rescued from apoptosis by cortisol-treatment (Weyts *et al.*, 1998b,a). The differential response after immune stimulation probably reflects the high sensitivity of splenic B-lymphocytes, due to their high constitutive GR2 expression, compared to head kidney phagocytes or thymus T-lymphocytes.

In conclusion, we show that depending on the type of immune stimulus and the cell type investigated, Hsp70 and the distinct GR variants are differentially regulated *in vitro* as well as *in vivo*. Together these molecules support a balanced sequential pro-inflammatory and anti-inflammatory cytokine expression profile required for effective, immune response.

Ackowledgements

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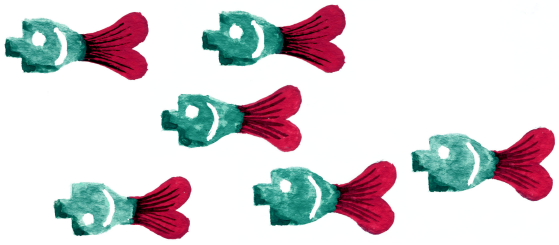

'See first, think later, then test. But always see first.
Otherwise you'll only see what you were expecting.'
Douglas Adams, So long, and thanks for all the fish

6

Differential gene expression in the head kidney of common carp (*Cyprinus carpio* L.) following restraint stress or infection, revealed by transcriptome analysis

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in preparation

Abstract

Restraint stress and *Trypanoplasma borreli* parasitaemia in common carp (*Cyprinus carpio* L.) were used as paradigms to define, by microarray, differentially expressed genes involved in stress and immune regulation. We analysed gene expression in head kidney tissue, that harbors both the interrenal cells and the major haematopoeietic cells. The parasitaemia induces a tightly controlled immune response with little interindividual variation, while the restraint stress results in a more variable gene expression profiles. Parasite infection consistently downregulated genes for the acute phase mediators lysozyme, granulin and C-type lectin and upregulated immunoglobulin light chain expression. Restraint increased expression of genes whose products are involved in metabolic processes towards energy release, protein synthesis, and muscle development and activation. We identified the hypothetical protein 'LOC406744' of the DUF727 protein family (a family of proteins with unknown function) and nephrosin, an astacin zinc-protease as genes that deserve focus in future stress and immune studies.

6.1 Introduction

The concept of allostasis, maintaining stability through change, is a physiological process through which organisms actively adjust to both abiotic and biotic (pathogenic) stressors (McEwen, 2007). The neuroendocrine and immune system of teleostean fishes have evolved in a balanced manner and exchange of information between these systems is crucial for allostasis. In studies on neuroendocrine-immune interactions in fish we often assume functional orthology and scan databases of mammals for genes involved in neuroendocrine regulation to define orthologous genes in the fish. However, this method is biased as mammals such as mice, rats and humans are highly specialised and ancestral regulatory systems present in teleostean fishes may easily be overlooked. Moreover, specific stress or immune genes that have evolved in teleostean fishes, but not in mammals, may also be missed. Therefore an unprejudiced approach to find (ancestral) genes involved in neuroendocrine-immune regulation is warranted. The use of microarray for genome wide expression profiling allows for simultaneous analysis of the expression of thousands of genes. The availability of the genomes of pufferfish (*Takifugu rubripes*), the green spotted puffer (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), ricefish (*Oryzias latipes)*, and stickleback (*Gasterosteus aculeatus*)) greatly facilitates the annotation of newly discovered genes on the basis of sequence homology. This approach yields an 'unbiased' identification of differentially expressed genes involved in stress adaptation by comparison of the genome of control and experimental fish.

Stress has profound effects on the immune response of fish, but the impact depends on the type and duration of the stressor. In carp, acute stress inhibits apoptosis in neutrophils, and induces release of head kidney neutrophilic granulocytes into circulation (Huising *et al.*, 2003a; Weyts *et al.*, 1998b). Prolonged stress decreases pro-inflammatory cytokine expression and induces apoptosis in B-lymphocytes and inhibits antibody production (Engelsma *et al.*, 2003a; Weyts *et al.*, 1998a). These effects are at least partly mediated by cortisol via glucocorticoid receptors (GRs) and heat shock protein 70 (Hsp70) and catecholaminergic pathways (adrenaline). Interestingly, the immune system appears to respond to endocrine control with a balanced pro- and anti-inflammatory cytokine response through differential regulation of the GRs and Hsp during the initial innate and the following adaptive response (Rhen and Cidlowski, 2005; Wang *et al.*, 2001).

Here we search for other, possibly interconnected, regulatory systems involved in regulation of the stress or the immune response. Restraint stress (constraint of the fish in a net for 24 hr) is a relatively mild acute stressor and is characterised by increased plasma cortisol and glucose levels; the animals are not physically harmed. Infection with the blood

parasite *Trypanoplasma borreli* causes prolonged and severe immune stimulation, which results in high parasitaemia, high levels of pro-inflammatory cytokines (Joerink *et al.*, 2007) and clinical signs of anaemia, splenomegaly, and enlarged head kidney (Bunnajirakul *et al.*, 2000) and might even be fatal (Forlenza *et al.*, 2008b). We focused our investigation on the head kidney. The head kidney of teleostean fishes harbors both endocrine (the interrenal endocrines) and immune cell lines: haematopoietic cells (formation of immune cells) and endocrine cells (production of stress hormones cortisol and adrenalin) are found in close vicinity in this organ. The head kidney therefore is a logical candidate for the study of neuroendocrine-immune interaction. The two paradigms elicit widely different responses and shed light on the robustness of the carp adaptability.

6.2 Experimental procedures

6.2.1 Animals

Nine month old carp (150–200 g) (*Cyprinus carpio* L.) were kept at 23 °C in recirculating UV-treated tap water at the 'De Haar Vissen' facility in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7 % of their estimated body weight. The cross 'R3×R8' is offspring of Polish (R3) and Hungarian (R8) strains (Irnazarow, 1995). All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

6.2.2 Tissue preparation

Fish were anaesthetised with 0.2 g/l tricaine methane sulfonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l $NaHCO_3$ (Merck, Darmstadt, F.R. Germany). Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe with a 21 gauge needle. Next, fish were killed by spinal transection and organs and tissues were carefully removed, snap frozen in solid CO_2 or liquid N_2 and stored at -80 °C for RNA extraction.

6.2.3 Restraint-stress paradigm

Restraint (24 hr) was given by netting the fish and suspending the nets with the fish in the tanks (Huising *et al.*, 2004a). After 24 hr, the experimental group was transferred at once to a tank with 0.2 g/l TMS, resulting in rapid (< 1 min) and deep anaesthesia prior to blood

sampling and killing. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anaesthesia (again within 1 min), immediately before sampling of the experimental group. Blood and organs were isolated as mentioned above.

6.2.4 Blood parasite infection.

Fish were infected with the blood parasite *Trypanoplasma borreli* (cloned as described by Steinhagen *et al.* (1989)), by intraperitoneal injection of 1×10^4 parasites per fish in 100 µl RPMI, or fish were injected with 100 µl RPMI (controls). Parasitaemia was measured using a Bürker counting chamber.

6.2.5 RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen, Carlsbad, CA, USA), as suggested by the manufacturer. Total RNA was precipitated in isopropanol, washed with 75 % ethanol and dissolved in nuclease-free water. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5 % agarose gel before proceeding with cDNA synthesis.

6.2.6 cDNA synthesis

One microliter of oligo dT (5.0 µg) was added to 10 µg total RNA and samples were incubated for 10 min at 70 °C and subsequently chilled on ice for 5 min. Next samples were left to acclimatise to room temperature. To each sample, 2 µl 10× reverse transcriptase buffer, 1 µl aa-dUTP/dNTP mix (20× 1:1, Sigma-Aldrich, Zwijndrecht, The Netherlands), 2 µl 0.1 M dithiothreitol (DTT) and 1.5 µl stratascript (Stratagene) were added and the mix was incubated for 1 hr at 48 °C. Subsequently, 10 µl of stop solution (900 µl 0.5 M EDTA and 100 µl 10 N NaOH) was added and the mix was incubated for 15 min at 65 °C, after which 30 µl of neutralisation buffer (75 µl 1 M HEPES, pH 7.4 with 25 µl 3 M NaOAc). Subsequently, reactions were cleaned using Nucleospin columns (Macherey-Nagel, Abgene).

6.2.7 Dye coupling

cDNA samples were resuspended in 5 µl 0.1 M $NaHCO_3$. For each reaction, 5 µl Cy dye (Amersham) was added. Typically Cy3 is used for the reference sample (comprised of all

treated and non-treated animals of either of the experiments) and Cy5 for the experimental sample) but dye swaps were included for every reaction. Reactions were incubated at room temperature for 1 hr in the dark. Unreacted dye was quenched by adding 6 µl of 4 M hydroxylamine to each sample. Subsequently samples were combined and cleaned using Nucleospin columns (Macherey-Nagel, Abgene), eluting with 50 µl of NE buffer.

6.2.8 Hybridisation

The carp microarray was constructed from 13,349 PCR-amplified cDNA clones, spotted onto poly-L-Lysine-coated glass slides (Gracey *et al.*, 2004). Eluted, purified samples were added to 25 µl of 3× hybridisation buffer (9× SSC, 0.6 % SDS, 60 mM HEPES). The resulting probe mix was denatured by incubation for 2 min at at 100 °C. Probes were applied to the slides and slides were covered with a lifterslip (VWR, Lutterworth, UK). Slides were placed overnight in a pre-warmed hybridisation chamber at 65 °C. The next day, slides were washed gently for 5 min with pre-warmed (65 °C) 1× SSC, 0.1 % SDS followed by an additional wash with pre-warmed 0.1× SSC for 5 min. Next slides were spinned dry for 5 min at 1000 rpm and scanned with a Axon GenePix® scanner. Scanned TIFF images were analysed using GenePix® 5.0 and results were saved as GenePix Result (*.gpr) files.

6.2.9 Data normalisation

R/Bioconductor (Gentleman *et al.*, 2004) package limma (Smyth, 2005) was used to analyse array data. The *.gpr files were imported into R (version 2.6.1), median values of both foreground and background intensities were extracted and used in the following analysis. Any spot with FLAG-value less than -50 was given a zero weight; spots with signal to background ratio (R/Rb and G/Gb) in both red and green channels less than 1.3 were given weight 0.1. We used background correction option 'normexp+offset' (offset = 50) (Ritchie *et al.*, 2007) background corrected data were normalised using 'printtiploess' normalisation (within array normalisation) followed by 'quantile' normalisation (between array normalisation) (Smyth and Speed, 2003). Array quality weight was calculated for each array using the method described previously by Ritchie *et al.* (2006). All control spots on the array were removed before fitting in the linear model and differentially expressed genes were identified using the method as described by Smyth (2004), multiple testing were corrected using False Discovery Rate (FDR) control method described by Benjamini and Hochberg (1995).

6.2.10 Differentially regulated genes

The randomly picked carp cDNA clones (Gracey *et al.*, 2004) were checked and 9202 high quality expressed sequence tags (ESTs) were submitted to EST open database dbEST and results are shown on the carpBASE-Browser. We used the Laboratory for Environmental Gene Regulation (LEGR) annotation database; carpBASE 2.1 (Sep. 2005) from the School of Biological Sciences of Liverpool University for analysis of differentially expressed genes http://legr.liv.ac.uk/searchDB/search.htm.

6.3 Results

6.3.1 Acute stress response

Restraint resulted in a classical stress response, indicated by significantly increased plasma cortisol and glucose levels compared to control fish (table 6.1). Plasma sodium (Na^+) levels had significantly decreased. Haematocrit did not change compared to control after restraint. To investigate the effect of restraint on gene expression we hybridised samples of 6 individual stressed and 5 individual control animals to a common reference in which all 11 animals were equally represented. Only 211 spots (2.3 %) met our stringency requirements of a false discovery rate (FDR) smaller than 0.05, (which corresponded to a raw P-value < 0.0001). The volcano-plot shows that relatively few genes are differentially expressed, but fold-changes are relatively high (figure 6.1).

For a first analysis, we accepted genes that showed < 0.7- or > 1.4-fold gene expression compared to the common reference, which corresponded to 107 differentially expressed genes (table 6.2). Of these differently expressed genes, 42 were annotated in carpBASE 2.1, but two genes were represented more than once: troponin, (2x) and GAPDH (2x).

Table 6.1: Plasma cortisol, glucose and sodium values and percentage of haematocrit after 24 hr restraint stress. Plasma samples of 7 control and 8 stressed fish were analysed. Data are plotted as average ± standard deviation and differences between groups were analysed by a students t-test. ** represents $P < 0.01$.

	Control	24 hr restraint
Plasma cortisol (ng/ml)	33.5 ± 22.2	235.6 ± 89.7 **
Plasma glucose (mmol/l)	2.6 ± 0.9	17.5 ± 4.1 **
Plasma sodium (mmol/l)	137.1 ± 2.7	127.0 ± 2.5 **
Haematocrit (%)	35.9 ± 3.7	38.4 ± 2.2

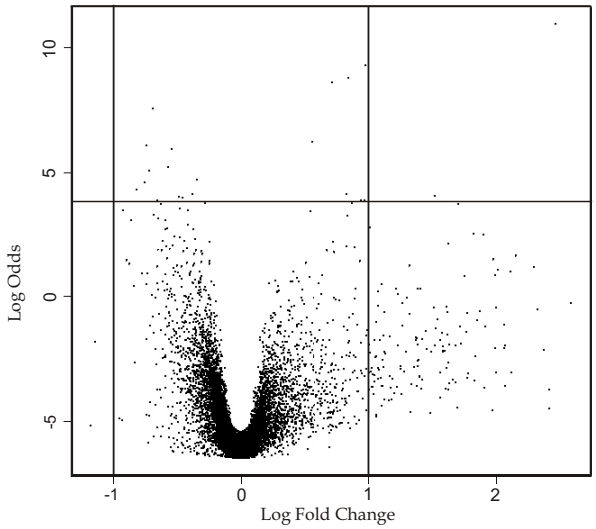


Figure 6.1: Volcano-plot (identifies significance and magnitude of change in expression of a set of genes between two conditions) of 24 hr restraint stress. The volcano displays the B-value or the log-odds on the y-axis, which represents the probability that a gene is differentially expressed. On the x-axis, the log 2 fold change in gene expression between two conditions is shown, which represents the magnitude of the response. Lines corresponding to the FDR < 0.05 (horizontally) and the genes with expression < 0.5 ($\log 2 < -1$) or > 2.0 ($\log 2 > 1$) compared to the common reference (vertically) are shown.

Differentially expressed genes with < 0.5- or > 2-fold gene expression compared to the common reference, were studied in further detail (table 6.3). No gene expression levels smaller than 0.5 were detected. Upregulated genes were involved in transcription and protein synthesis or modification (Y-box protein, ribosomal protein and ubiquitin), glucose metabolism (glycogen phosphorylase, GAPDH), or muscle development and activity (parvalbumin troponin, and creatin kinase). The strongest upregulated gene (6×) was a hypothetical protein 'LOC406744' (from a family of proteins with unknown function, DUF727).

6.3.2 Parasite infection

Plasma immune and stress parameters were measured 3 weeks after infection with *Trypanoplasma borreli* (table 6.4). After parasite infection plasma cortisol values were increased, but not significantly due to high variability, and plasma glucose values were unaffected. Parasite infection resulted in anaemia indicated by decreased haematocrit. Leukocrit was significantly increased in parasite infected fish, indicative of a strong immune response. To investigate the effect of *T. borreli* infection on gene expression we hybridised samples of 4 individual infected, and 4 individual control animals to a common reference in which all 8 animals were equally represented. Only 215 spots (2.4 %) met our stringency requirements

Table 6.2: Differentially expressed genes. Upregulated genes (a) and downregulated genes (b) after 24 hr of restraint by netting. Only genes with expression < 0.7 or > 1.4 compared to the common reference are shown. GenBank accession numbers and fold change in gene expression compared to common reference is shown. **Note:** genes with expression < 0.5 or > 2.0 compared genes are omitted as they are shown in table 6.3.

(a) Upregulated genes

Gene	Acc. No.	Fold change
Troponin T3b, skeletal, fast	NP_857636	1.46
Protein phosphatase 2	NP_998541	1.49
DNA helicase ERCC2/XPD	I51720	1.53
Vitellogenin	AAL07472	1.53
Matrix metalloproteinase 13	AAQ07962	1.60
Retinol dehydrogenase 1	NP_955903	1.71
ATPase, H^+ transporting, lysosomal	NP_775362	1.78
Hypothetical protein LOC436628	NP_001002355	1.80
Hypothetical protein LOC449781	NP_001005954	1.86
Creatine kinase M3-CK	AAC96094	1.86
Zgc:103434	AAH83378	1.87
CTL2 gene	XP_686419	1.92
Bromodomain, testis-specific	XP_706974	1.97

(b) Downregulated genes

Gene	Acc. No.	Fold change
Heat shock / stress protein HSP90-beta	AAD50973	0.54
GABA-RAP (GABA-receptor-associated protein)	XP_694222	0.56
Hypothetical protein LOC541490	NP_001014325	0.62
ATPase, H^+ transporting	BC056822	0.63
Adenine nucleotide translocator s254	BAD86710	0.64
Constitutive heat shock protein HSC70-2	AAP51388	0.64
Guanine nucleotide binding protein (G protein)	BC049459	0.64
Allantoicase	NP_001002716	0.66
GAPDH protein	AAH95386	0.66
Zinc finger protein 207	BC065456	0.67
Glutamate oxaloacetate transaminase 2	NP_956283	0.67
Tocopherol (alpha) transfer protein	NP_956025	0.67
Tetratricopeptide repeat domain 13	XP_696659	0.68
Dihydrofolate reductase (DHFR)	AAH71330	0.69
Myosin binding protein C, fast type	NP_004524	0.69
Myosin VIIa	NP_694515	0.70

Table 6.3: Differentially expressed genes. Upregulated genes (a) and downregulated genes (b)after 24 hr of restraint by netting. Only genes with expression < 0.5 or > 2.0 compared to the common reference are shown GenBank accession numbers, fold change in gene expression compared to common reference, and gene ontology is shown. **Note:** no downregulated genes were detected.

Upregulated	Acc. No.	Fold change	Gene ontology
Y box protein 1	BAA19849	2.02	GO:0003676, nucleic acid binding; GO:0003677, DNA binding; GO:0006355, regulation of transcription
Carboxylesterase 2	XP_690455	2.11	
Parvalbumin	CAC83659	2.15	GO:0005509, calcium ion binding; GO:0007517, muscle development
Thioredoxin domain	NP_001003456	2.33	
Ribosomal protein L21	XP_689959	2.50	GO:0003723, RNA binding; GO:0003735, structural constituent of ribosome; GO:0006412, protein biosynthesis
Glycogen phosphorylase, muscle	BC055181	2.62	
GAPDH protein	AAH95386	2.66	GO:0008943, glyceraldehyde 3-phosphate dehydrogenase activity; GO:0006006, glucose metabolism; GO:0006096, glycolysis
Solute carrier family 41	XP_682785	2.87	
Troponin T3b, skeletal, fast	NP_857636	3.25	GO:0007517, muscle development
Myelin basic protein	AAW52552	3.54	
Creatine kinase M2-CK	AAC96093	3.74	GO:0004111, creatine kinase activity; GO:0016301, kinase activity; GO:0016740, transferase activity; GO:0016772, transferring phosphorus-containing groups
Ubiquitin C variant	BAD93019	4.32	GO:0006464, protein modification
Hypothetical protein LOC406744	NP_998600	6.00	

of a false discovery rate (FDR) smaller than 0.05 (which corresponded to a raw $P-$value < 0.0001). The volcano-plot shows a stable expression pattern of differentially expressed genes, and fold changes are relatively low (figure 6.2).

For a first analysis, we accepted genes that showed < 0.7- or > 1.4-fold gene expression compared to the common reference, which corresponded to 128 differentially expressed genes (table 6.5). Of these differently expressed genes, 79 were annotated in carpBASE 2.1, but several genes were represented more than once; aldolase (7×), granulin (7×), tubulin (4×) haemoglobulin (2×), calmodulin (2×) cofilin (2×), lysozyme C (2×) and constitutive heat shock protein HSC70-2 (2×).

Differentially expressed genes with < 0.5- or > 2-fold gene expression compared to the common reference, were studied in further detail (table 6.6). The genes for immunoglobulin

Table 6.4: Plasma cortisol, glucose and sodium values and percentage of haematocrit and leukocrit after parasite infection. Plasma samples of 6 control and 6 stressed fish were analysed. Data are plotted as average ± standard deviation and differences between groups were analysed by a students *t*-test. * represents $P < 0.05$ and ** represents $P < 0.01$. **Note:** one control fish had a very high plasma cortisol level and is depicted in brackets.

	Control	Parasite infection
Plasma cortisol (ng/ml)	37.1 ± 10.9 (487.1)	216.7 ± 287.3
Plasma glucose (mmol/l)	3.7 ± 0.7	3.3 ± 0.9
Plasma sodium (mmol/l)	140.8 ± 3.9	136.8 ± 4.7
Haematocrit (%)	37.7 ± 2.7	26.7 ± 4.0 **
Leukocrit (%)	0.9 ± 0.3	1.6 ± 0.3 *

light chain and tropomyosin were both 3-fold increased. The immune mediators granulin (represented 5×), C-type lectin (represented 4×) and lysozyme were downregulated, with expression levels of 0.4, 0.3 and 0.4 respectively, compared to the common reference. Gene expression of nephrosin precursor had also decreased, 0.5 compared to the common reference.

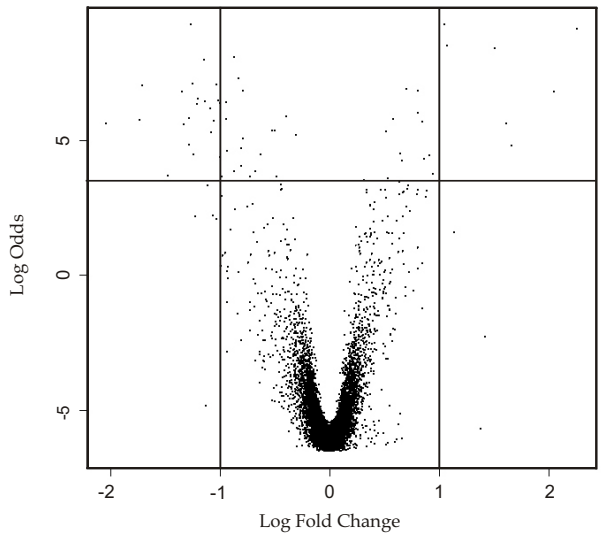


Figure 6.2: Volcano-plot of parasite infection. The volcano displays the B-value or the log-odds on the y-axis, which represents the probability that a gene is differentially expressed. The log2 fold change in gene expression between two conditions is shown on the x-axis, which represents the magnitude of the response. Lines corresponding to the FDR < 0.05 (horizontally) and the genes with expression < 0.5 ($\log 2 < -1$) or > 2.0 ($\log 2 > 1$) compared to the common reference (vertically) are shown.

Table 6.5: Differentially expressed genes. Upregulated genes (a) and downregulated genes (b) after 3 weeks of parasite infection. Only genes with expression < 0.7 or > 1.4 compared to the common reference are shown. GenBank accession numbers and fold change in gene expression compared to common reference is shown. **Note:** genes with expression < 0.5 or > 2.0 compared genes are omitted as they are shown in table 6.3.

(a) Upregulated genes

Gene	Acc. No.	Fold change
Cofilin 2, like (28) (2×)	NP_998806	1.42–1.56
Solute carrier family 25	NP_775354	1.42
Aldolase A, fructose-biphosphate (7×)	NP_919358	1.43–1.93
Signal sequence receptor	XP_685191	1.43
Y box binding protein 1	BC050156	1.43
Der1-like domain family	BC045413	1.46
GAPDH protein	AAH95386	1.46
Calmodulin 2a (phosphorylase kinase) (2×)	BC059427	1.47–1.59
Proteasome (prosome, macropain)	CAD87789	1.47
Constutitive heat shock protein Hsc70-2 (2×)	AAP51388	1.49–1.80
Phosphoglycerate mutase 2 (muscle)	NP_957318	1.49
Der1-like domain family	BC045413	1.50
Tubulin, alpha	BC059428	1.54
Paf1/RNA polymerase II complex component	BC053163	1.56
Armadillo repeat	XP_700221	1.59
Mitochondrial ATP synthase	BAB47389	1.60
Tubulin, beta (4×)	BC062827	1.60–1.81
Elongation factor 1-alpha	AAO49408	1.62
Triosephosphate isomerase (2×)	BC053294	1.63–1.75
Junctophilin 1	XP_707181	1.64
COP9 constitutive photomorphogenic protein	BC06561	1.65
Cold inducible RNA binding protein	NP_956311	1.67
Proteasome (prosome, macropain) 3	NP_001006491	1.74
ATP synthase subunit beta, mitochondrial precursor	Q9PTY0	1.75
ATP synthase subunit gamma mitochondrial	BAB47390	1.75
Signal sequence receptor, delta	NP_001002082	1.83
Proteasome (prosome, macropain) 7	NP_998331	1.85

(b) Downregulated genes

Gene	Acc. No.	Fold change
C-type lectin	BAA95671	0.50
Mannose binding-like lectin precursor	AAF63468	0.51
Granulin 3 (7×)	P81015	0.52–0.65
Hemoglobin subunit alpha (2×)	P02016	0.52–0.62
Hemoglobin subunit beta	BAA20516	0.55
Mmp13 protein (2×)	XP_685843	0.57–0.70
Lysozyme C (2×)	BAA95698	0.58–0.63
CD63 antigen	NP_955837	0.59
Myeloid protein-1	BAB16024	0.60
Ribosomal protein S7	BC059562	0.60
FLAP (5-lipoxygenase activating protein)	XP_700090	0.67
Tumor differentially expressed 2 protein	XP_697810	0.68
Selenoprotein P	BC059656	0.68
Mapre1l protein	XP_706909	0.70

Table 6.6: Differentially expressed genes after 24 hr of restraint by netting. Only genes with expression < 0.5 or > 2.0 compared to the common reference are shown. GenBank accession numbers, fold change in gene expression compared to common reference, and gene ontology is shown.

(a) Upregulated genes

Gene	Acc. No.	Fold change	Gene ontology
Immunoglobulin light chain	AAG31727	2.84	
Tropomyosin	P84335	3.16	GO:0003779, actin binding

(b) Downregulated genes

Gene	Acc. No.	Fold change	Gene ontology
Granulin (5×)	P18015	0.40–0.50	GO:0005125, cytokine activity
C-type lectin (4×)	BAA95671	0.24–0.46	GO:0005529, sugar binding
Nephrosin precursor	AAB62737	0.47	GO:0008237, metallopeptidase activity; GO:0008270, zinc ion binding; GO:0008533, astacin activity
Lysozyme	BAA95698	0.42	GO:0003796, lysozyme activity; GO:0003824, catalytic activity; GO:0016787, hydrolase activity

6.4 Discussion

The high stringency applied in our microarray approach to identify differential gene expression provides strong evidence that the genes identified are indeed consistently involved in the stress and/or immune responses evoked among the individual fish. This notion was corroborated by the consistent responses of several genes (granulin, and C-type lectin) that were represented by multiple clones on the array and showed very constant responses. Clearly, the stringent analysis required for reliability, focus on the head kidney only, and the time points chosen, may have prevented us from finding all differentially regulated and physiologically relevant genes.

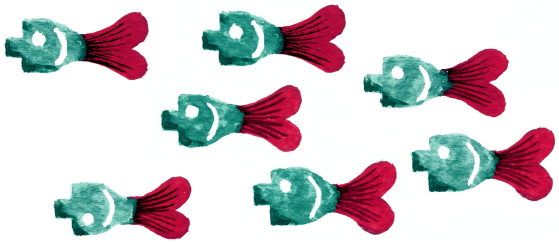
The volcano-plots showed very different responses after restraint or infection. After restraint many genes had at least doubled in expression, but were not differentially expressed according to statistical criteria, most likely due to interindividual variation. We hypothesise that the individual fish may upregulate specific genes, but that the strength of the response is variable, in line with individual coping strategies to the stressor (Korte *et al.*, 2005). Future research should address high- and low-responders to stress and investigate the differentially regulated genes of groups and individuals. The parasite infection gave a

more uniform response with approximately equal numbers of up- and downregulated genes and a similar response among individuals. The severity of this potentially lethal parasite infection apparently evokes a more consistent reaction of key differentially regulated genes, which together create a balanced and effective immune response.

Restraint stress increased expression of genes involved in metabolic processes, coupled to release of energy (Mommsen *et al.*, 1999; Wendelaar Bonga, 1997): glycogen phosphorylase (2.6-fold increased) is required to convert glycogen to glucose (Roach, 2002) and GAPDH (2.7-fold increased) generates eventually ATP via the process of glycolysis. Interestingly, expression of genes involved in muscle maintenance and activity (creatine kinase, parvalbumin and troponin) had also increased, although head kidney clearly has no muscle function. To regulate the demand for newly synthesised proteins (as the ones mentioned above), various components of the protein synthesis machinery, such as transcription factor Y-box protein, (3.7-fold increased), ribosomal protein (2.5-fold increased) and ubiquitin (4.3-fold increased) were upregulated. Together this suggests a response consistent with the fight or flight paradigm (McEwen, 2007), the animal has prepared itself but a dedicated response to avoid or neutralise the stressor is not possible. We assume that three weeks after start of infection with the blood parasite *T. borreli*, the acute phase response will be (partly) inhibited, and the adaptive immune response operational. Indeed, an upregulation of the immunoglobulin light chain was found , whereas genes for mediators of the innate immune system (acute phase proteins) such as growth factor granulin (Hanington *et al.*, 2006), lytic enzyme lysozyme (Magnadottir, 2006) and C-type lectin, involved in opsonisation, phagocytosis or activation of complement (Magnadottir, 2006), were downregulated. Antibody production in carp following infection is initiated after three weeks (Joerink *et al.*, 2007; Saeij *et al.*, 2003a), but further research into additional determinants of the innate and adaptive immune system and additional time points during infection is indicated and warranted.

After restraint stress, a six-fold increase was seen for the gene coding for a hypothetical protein, LOC406744. A similar protein is found in many vertebrate and invertebrate species, but so far no function has been established for this member of the DUF727 protein family. It was reported to be upregulated in holocarboxylase synthetase (HCS)-deficient *Drosophila melanogaster*, possibly to compensate for the decrease in histone biotinylation (required for heterochromatin structures, DNA repair, and mitotic chromosome condensation) (Camporeale *et al.*, 2006). As this protein family is found in such distantly related species it is tempting to hypothesise that these genes serves a very fundamental function in (cell) physiology. After parasite infection nephrosin shows a similar expression profile as

the acute phase mediators granulin, lysozyme and C-type lectin. Nephrosin is a member of the astacin family (Hung *et al.*, 1997). It is a secreted zinc-dependent proteinase present in immune organs in fish, but also has molecular characteristics similar to hatching enzymes (Kawaguchi *et al.*, 2006; Tsai *et al.*, 2004). It is suggested to play a role in haematopoiesis and appears relatively late stage marker of granulocytic differentiation (Song *et al.*, 2004). It is suggested to be involved in the immune response as it was found to be upregulated after lipopolysaccharide (LPS) expression (Darawiroj *et al.*, 2008). Interestingly, it was also found to be upregulated in the gills of saltwater-acclimatised fish, which could reflect an effect of hormones involved in osmoregulation, such as prolactin (PRL) and growth hormone (GH) that are known to enhance immune functions in fish (Boutet *et al.*, 2006). Nephrosin therefore could be a good example of an immune gene under neuroendocrine control.



'It's a very funny thing [...] but there seem to be two now. This -whatever-it-was- has been joined by another -whatever-it-is- and the two of them are now proceeding in company.'

A.A. Milne, The complete Winnie-the-Pooh

7

Differential expression of two interferon-γ genes in common carp (*Cyprinus carpio* L.)

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Developmental and Comparative Immunology **(2008), 32(12), 1467–1481**[1]

[1]

Abstract

Two interferon gamma (IFN-γ) genes are expressed in immune cells of teleostean fishes and are potentially implicated in B- and T-lymphocyte responses. IFN-γ-2 shows structural and functional characteristics to other vertebrate IFN-γ genes and is associated with T-lymphocyte function. Expression profiling shows IFN-γ-2 upregulation in T-lymphocytes after phytohaemagglutinin (PHA)-stimulation *in vitro*. Unexpectedly, we found IFN-γ-1, which is structurally different from IFN-γ-2, to be expressed in lipopolysaccharide (LPS)-stimulated IgM^+ (B- lymphocyte enriched) fractions. Expression of T-box transcription factor T-bet, but not of GATA binding protein 3 (GATA3), correlated with expression of both IFN-γ genes. *In vivo* parasite infection, but as predicted not zymosan-induced inflammation, resulted in concomitant upregulation of T-bet and IFN-γ-2. This corroborates a genuine T-lymphocyte cell associated role for IFN-γ-2.

7.1 Introduction

Interferon gamma (IFN-γ) is a key cytokine for innate and adaptive immunity against viral and intracellular bacterial infections and involved in tumor control. It is only active as a homodimer (Rosenzweig and Holland, 2005; Schroder *et al.*, 2004; Walter *et al.*, 1995). IFN-γ stimulates macrophage-mediated phagocytosis and production of pro-inflammatory cytokines and anti-microbial oxygen radicals by macrophages (Schoenborn and Wilson, 2007). In mammals IFN-γ is constitutively produced by natural killer (NK) cells of the innate arm of the immune system (Stetson *et al.*, 2003), whereas T-lymphocytes of the adaptive arm produce IFN-γ after activation or differentiation into effector T-cells in response to IL-12 and IL-18 (Glimcher *et al.*, 2004). Although different regulatory regions of the IFN-γ locus have been identified, expression is primarily regulated by two transcription factors, GATA-binding protein 3 (GATA3) and a T-cell member of the T-box family; T-bet (Szabo *et al.*, 2000; Zheng and Flavell, 1997). T-bet is involved in chromatin remodelling of the IFN-γ gene to allow for IFN-γ transcription, whereas GATA3 inhibits IFN-γ expression (Murphy and Reiner, 2002; Zheng and Flavell, 1997).

The pleiotropic and redundant character of the cytokine signal family reflects its complex and subtle regulatory functions and may be at the basis of the phenomenal radiation of these signal molecules in vertebrates. Transcription factors, necessary for intracellular signaling and driving cytokine expression, execute specific functions of vital importance which elicits greater purifying selection. This is reflected by low sequence identity between cytokines of different, especially distantly related, species and high sequence conservation among transcription factors. This complicates finding orthologues of cytokine genes in representatives of evolutionary ancient species. Additionally, these orthologues might have unexpected functions.

Recently, IFN-γ was described in at least three fish species. As predicted (see above) sequence similarity is low, less than 25 %, compared to mammalian IFN-γ, but the typical cytokine features, such as instability motifs, gene structure, and predicted three-dimensional protein structure are comparable (Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006; Zou *et al.*, 2005). In channel catfish (*Ictalurus punctatus*), puffer fish (*Tetraodon nigroviridis*) and zebrafish (*Danio rerio*), a second IFN-γ gene was found, that may be the result from a tandem duplication (Igawa *et al.*, 2006). In all species with duplicate IFN-γ genes, IFN-γ-2 shares more structural similarities with known vertebrate IFN-γ genes, including the human IFN-γ protein. Indeed also our common carp IFN-γ-2 shows typical features such as a comparable signal peptide, the IFN-γ signature motif ([I/V]-Q-X-[K/Q]-A-X_2-E-[L/F]-X_2-

[I/V]), mRNA instability motifs and a predicted 6 helices secondary structure as described for human IFN-γ (Ealick *et al.*, 1991). IFN-γ proteins have a highly and basic hydrophilic C-terminus and a nuclear localisation sequence (NLS) of four contiguous basic amino acids that are required for IFN-γ function (Zou *et al.*, 2005). The common carp NLS consists of four arginine residues as holds for the proteins of channel catfish, zebrafish, and puffer fish (Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006). In zebrafish and channel catfish constitutive IFN-γ mRNA expression for both genes was demonstrated in several immune tissues and cell types and this expression is regulated by immunostimulants. In these species IFN-γ constitutive mRNA expression of both genes was found in several immune organs and cell types, and this expression could be regulated by immune stimulants (Milev-Milovanovic *et al.*, 2006).

Bony fishes represent the earliest true vertebrates with a well-developed innate and adaptive immune system. The finding of two types of IFN-γ genes prompted us to search for possible ancestral functions. 'Master regulators' for IFN-γ expression, T-bet and GATA3, were recently described in T-lymphocyte enriched lymphocyte fractions of the ginbuna crucian carp (*Carassius auratus langsdorfii*) (Takizawa *et al.*, 2008a,b). We proceeded to define these genes in common carp which gave us a unique opportunity to investigate IFN-γ function. We show differential expression of the two IFN-γ genes and the regulatory transcription factors T-bet and GATA3 by LPS- and PHA-treatments and in relation to thymocyte maturation status *in vitro*. Moreover, we determined expression profiles in an inflammation and an infection paradigm *in vivo*. Genuine IFN-γ functions were corroborated for teleostean fishes but interestingly, were executed by two different IFN-γ genes.

7.2 Experimental procedures

7.2.1 Animals

Common carp (*Cyprinus carpio* L.) were kept at 23 °C in recirculating UV-treated tap water at 'De Haar Vissen' in Wageningen. Fish were fed dry food pellets (Promivi, Rotterdam, The Netherlands) at a daily maintenance ration of 0.7 % of their estimated body weight. The cross 'R3×R8' is offspring of Hungarian (R8) and Polish (R3) strains (Irnazarow, 1995). Experimental repeats were performed with fish from different batches of eggs. All experiments were performed according to national legislation and were approved by the institutional Ethical Committee.

7.2.2 Identification of IFN-γ genes

We incorporated IFN-γ-1 and IFN-γ-2 sequences described for zebrafish, channel catfish, pufferfish and rainbow trout as well as several mammalian IFN-γ sequences in separate multiple sequence alignments for IFN-γ-1 and IFN-γ-2 , using CLUSTALW (Chenna *et al.*, 2003). Primers were designed in regions of high amino acid identity. We obtained partial cDNA sequences from a λZAP cDNA library of carp brain. By RACE (rapid amplification of cDNA ends; Invitrogen, Carlsbad, CA, USA) the corresponding full length sequences were obtained. PCR was carried out as previously described (Huising *et al.*, 2004b) and sequences were determined from both strands.

7.2.3 Tissue preparation

Nine months old carp (150–200 g) were anaesthetised with 0.2 g/l tricaine methane sulfonate (TMS) (Cresent Research Chemicals, Phoenix, AZ, USA) buffered with 0.4 g/l $NaHCO_3$. (Merck, Darmstadt, F.R. Germany). Blood was collected by puncture of the caudal vessels using a heparinised (Leo Pharmaceuticals Products, Ltd, Weesp, The Netherlands) syringe fitted with a 21 gauge needle. Next, fish were killed by spinal transection and organs and tissues for RNA extraction were carefully removed, snap frozen in solid CO_2 or liquid N_2 and stored at -80 ℃.

7.2.4 Cell collection

Gill and gut lymphocytes. Intestinal and branchial epithelia were collected by scraping the epithelia off the underlying connective tissue and branchial arches respectively, on an ice-cooled glass plate using a microscope slide. The tissues thus obtained were passed through a 100 µm nylon mesh (BD Bioscience, Breda, The Netherlands) with carp RPMI (cRPMI; RPMI 1640, Invitrogen Carlsbad, CA, USA; adjusted to carp osmolality (280 mOsm/kg) and washed twice. The cell suspension was layered on 3 ml Ficoll (density 1.077 g/ml, Amersham Biosciences, Uppsala, Sweden). Following subsequent centrifugation at 800 *g* at 4 ℃ for 25 min with the brake disengaged leukocytes at the interface were collected and washed twice with cRPMI and once with $cRPMI^{++}$ (cRPMI supplemented with 0.5 % pooled carp serum, 1 % glutamine (Cambrex, Verviers, Belgium), 1 % penicillin G (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1 % streptomycin sulphate (Sigma-Aldrich)).

Thymocytes were obtained by by passing the tissue through a 100 µm nylon mesh (BD Bioscience, Breda, The Netherlands) with cRPMI and washed twice. The cell suspension was layered on a discontinuous Percoll (Sigma-Aldrich) gradient (1.020, 1.060 and

1.070 g/cm^3) and centrifuged 30 min at 800 *g* with the brake disengaged. Cells with density 1.020–1.060 (predominantly mature thymocytes) and with density 1.060–1.070 g/cm^3 (predominantly immature thymocytes) (Rombout *et al.*, 1997) were collected and washed twice with cRPMI and once with cRPMI^{++}.

Anterior head kidney phagocytes were obtained by passing the tissue through a 100 µm nylon mesh (BD Bioscience) with cRPMI and washed twice. The cell suspension was layered on a discontinuous Percoll (Sigma-Aldrich) gradient (1.020, 1.060, 1.070 and 1.083 g/cm^3) and centrifuged 30 min at 800 *g* with the brake disengaged. Cells at the 1.070 and 1.083 g/cm^3 were collected and washed twice with cRPMI and once with cRPMI^{++}. Relative cell populations were found to be similar as described before; 1.070 g/cm^3 predominantly macrophages (65 %), with 10 % granulocytes and 25 % small macrophages and lymphocytes and 1.083 g/cm^3 predominantly neutrophilic granulocytes (85 %) with 15 % macrophages) interface (van Kemenade *et al.*, 1994).

PBL. To obtain peripheral blood lymphocytes (PBL), blood was centrifuged 5 min at 100 *g* and afterwards 10 min at 800 *g* at 4 °C. The buffy coat and a small amount of serum were mixed and loaded on 3 ml Ficoll (density 1.077 g/ml, Amersham Biosciences, Uppsala, Sweden). Following subsequent centrifugation at 800 *g* at 4 °C for 25 min with the brake disengaged leukocytes at the interface were collected and washed twice with cRPMI and once with cRPMI^{++}.

Magnetic Activated Cell Sorting (MACS) of PBL was performed as described before (Forlenza *et al.*, 2008a). Briefly, WCI-12, a mouse monoclonal antibody directed against the heavy chain of carp IgM was used to positively select B-lymphocytes bearing surface IgM, whereas the negatively selected fraction was enriched for T-lymphocytes. Purity assessed by flow cytometric analysis was > 90 % (WCI-12$^+$ fraction, IgM$^+$); the WCI-12$^-$ fraction contained < 25 % WCI-12$^+$ cells. The WCI-12$^-$ (IgM$^-$, T-lymphocyte-enriched) fraction expressed T-lymphocyte marker genes CD8-α, CD8-β and TCR-α (Forlenza *et al.*, 2008a).

7.2.5 *In vitro* stimulation

Cell stimulations were carried out *in duplo* in cRPMI^{++} at 5.5 × 10^6 cells per well (in 500 µl) in a 24 well cell culture plate. Cells were stimulated for 4 hr at 27 °C at 5 % CO_2 with 50 µg/ml LPS (Lipopolysaccharide from *Escherichia coli 055:B5, (L2880, purified by phenol extraction)* Sigma-Aldrich), 50 µg/ml Poly inosinic poly cytidiylic (Poly I:C, Sigma-Aldrich) or 10 µg/ml phytohaemagglutinin (PHA, Sigma-Aldrich) Control cells, received medium only, experiments were repeated for four independent fish. After stimulation supernatant was removed and cells were collected in 300 µl RLT buffer from the RNeasy Mini Kit

(Qiagen, Venlo, The Netherlands) (duplicate treatments were pooled) and stored at -80 °C.

7.2.6 Zymosan-induced peritonitis and blood parasite infection

A sterile, zymosan-induced peritonitis model was used as described before (Chadzinska *et al.*, 2008). Animals were either untreated (intact fish), sham injected, or i.p.-injected with freshly prepared zymosan A (2 mg/ml, 1 ml/50 g body weight; Sigma-Aldrich). At the selected time points animals were sacrificed and their peritoneal cavities were lavaged with 1 ml of ice cold PBS.

In a separate experiment fish were infected with the blood parasite *Trypanoplasma borreli* (cloned as described by Steinhagen *et al.* (1989), by intraperitoneal injection of 1×10^4 parasites per fish in 100 µl RPMI (n=4), or fish were injected with 100 µl RPMI (n=4; controls). Parasitemia was measured using a Bürker counting chamber.

7.2.7 RNA isolation

RNA was isolated from tissues after extraction in Trizol reagent (Invitrogen, Carlsbad, CA, USA), as suggested by the manufacturer. Total RNA was precipitated in isopropanol, washed with 75 % ethanol and dissolved in nuclease-free water. RNA of stimulated cells was isolated as described by the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) strictly according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry and integrity was ensured by analysis on a 1.5 % agarose gel before proceding with cDNA synthesis.

7.2.8 DNAse treatment and first strand cDNA synthesis

For each sample a '–RT' (non reverse transcriptase) control was included. One microliter of 10× DNase-I reaction buffer and 1 µl DNase-I (Invitrogen, 18068-015) was added to 1 µg total RNA and incubated for 15 min at room temperature in a total volume of 10 µl. DNase I was inactivated with 1 µl 25 mM EDTA at 65 °C for 10 min. To each sample, 300 ng random hexamers (Invitrogen, 48190-011), 1 µl 10 mM dNTP mix, 4 µl 5× First Strand buffer, 2 µl 0.1 M dithiothreitol (DTT) and 40 Units RNAse Out (Invitrogen 10777-019) were added and the mix was incubated for 10 min at room temperature and for and additional 2 min at 37 °C. To each sample (not to the '–RT' controls) 200 U Superscript-II RNase H^- Reverse Transcriptase (RT; Invitrogen, 18064-014) was added and reactions were incubated for 50 min at 37 °C. Demineralised water was added to a final volume of 100 µl and stored at -20 °C until further use.

Table 7.1: Primers used for gene expression studies. Table shows sequences and GenBank accession numbers.

Gene	FW primers	RV primers	Acc. No.
IFN-γ-1	TGC-ACT-TGT-CAG-TCT-CTG-CT	TGT-ACT-TGT-CCC-TCA-GTA-TTT	AM261214
IFN-γ-2	TCT-TGA-GGA-ACC-TGA-GCA-GAA	TGT-GCA-AGT-CTT-TCC-TTT-GTA-G	AM168523
GATA3	CTC-TTC-CTC-CTC-GCT-GTC-TG	ATG-AGC-CCG-AAC-CTG-ATG	AM947129
T-bet	ACC-GAA-CCG-CCT-TGA-CTT	TTT-TCA-GAG-TAG-TAG-CCC-AGA-GG	AM944367
β-Actin	GCT-ATG-TGG-CTC-TTG-ACT-TCG-A	CCG-TCA-GGC-AGC-TCA-TAG-CT	M24113
40S	CCG-TGG-GTGA-CAT-CGT-TAC-A	TCA-GGA-CAT-TGA-ACC-TCA-CTG-TCT	AB012087

7.2.9 Real-time quantitative PCR

PRIMER EXPRESS (Applied Biosystems, Foster City, CA, USA) and *PRIMER3* software (Rozen and Skaletsky, 2000) was used to design primers for use in real-time quantitative PCR (RQ-PCR) (table 7.1). For RQ-PCR 5 µl cDNA and forward and reverse primers (300 nM each) were added to 7 µl Brilliant® SYBR® QPCR Master Mix (Stratagene, La Jolla, CA, USA) and demineralised water was added to a final volume of 14 µl. RQ-PCR (10 min 95 ℃, 40 cycles of 15 s at 94 ℃, 30 s at 60 ℃, and 30 s at 72 ℃ followed by 1 min at 60 ℃) was carried out on a Rotorgene 2000 real-time cycler (Corbett Research, Sydney, Australia). Raw data were analysed with comparative quantitation of the Rotor-gene Analysis Software V5.0. Constitutive gene expression in organs and tissues was determined as a ratio of target gene *vs* reference gene and was calculated according to the following equation.

$$\text{ratio} = \frac{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}}}{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}}} \tag{7.1}$$

where E is the amplification efficiency and Ct is the number of PCR-cycles needed for the signal to exceed a predetermined threshold value. Expression following *in vitro* or *in vivo* stimulation was determined relative to the expression of non-stimulated cells or control fish according to the following equation (Pfaffl, 2001).

$$\text{ratio} = \frac{(\text{E}_{\text{target}})^{\text{Ct}_{\text{target}}(\text{control} - \text{sample})}}{(\text{E}_{\text{reference}})^{\text{Ct}_{\text{reference}}(\text{control} - \text{sample})}} \tag{7.2}$$

Dual internal reference genes (40S ribosomal protein and β-actin) were incorporated in all RQ-PCR experiments; results were similar following standardisation to either gene. '–RT'-controls were included in all experiments and no amplification above background levels was observed. Non-template controls were included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was

ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

7.2.10 Bioinformatics

Sequences were retrieved from the Swissprot, EMBL and GenBank databases using SRS and/or BLAST (basic local alignment search tool) (Altschul *et al.*, 1997). Multiple sequence alignments were carried out using CLUSTALW (Chenna *et al.*, 2003). Calculation of pairwise amino acid identities was carried out using the SIM ALIGNMENT tool (Huang and Miller, 1991). Protscale was used secondary structure (helix) prediction and generation of hydrophobicity plot (Gasteiger *et al.*, 2005). Signal IP was used for prediction of signal peptides (Bendtsen *et al.*, 2004). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004). A phylogenetic tree was constructed based on the neighbour-joining method using the Poisson-correction for evolutionary distance (Nei and Kumar, 2000). Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

7.2.11 Statistics

Statistic analysis was performed with SPSS 12.0.1 software. Following ANOVA, significance of differences between treatments was assessed by Mann-Whitney U-test, and $P < 0.05$ was taken as fiducial limit. Correlations were tested with Spearman's *Rho*-test $P < 0.05$ was accepted as fiducial limit. For RQ-PCR data, tests were performed for both internal reference genes (β-actin and 40S) and statistical significance is only reported if both reference genes showed a significant effect, where * indicates $P < 0.05$. Data of *in vitro* stimulation experiments are shown in box plots, where the box shows the interquartile range, the black line the median value and the whiskers the 10- and 90 % percentiles.

7.3 Results

Identification of two common carp IFN-γ. Carp IFN-γ genes were amplified using a set of primers targeted to conserved regions within other fish IFN-γ genes. The first full-length cDNA sequence translated into a precursor protein of 170 amino acids, with a predicted signal peptide of 26 amino acids (figure 7.1a). This sequence showed 15–20 % amino acid identity to non-fish vertebrate IFN-γ sequences. However, moderate amino acid identities (45–50 %) were found when comparison was made to channel catfish or zebrafish IFN-γ-1

```
tccaagctggtttacagctgtgtgtttagcaaaaatgtattgttggcttaacatggtgcac
                                  M  Y  C  W  L  N  M  V  H
ctgatatgtgcacttctcctaatagtgtctctgcaaggaaccgtcggagccagacttcca
 L  I  C  A  L  L  L  I  V  S  L  Q  G  T  V  G  A  R  L  P
cagtcccaaaatgacaaagagcaaatgctgaagaacttaagagaaaaaatcgaacctctg
 Q  S  Q  N  D  K  E  Q  M  L  K  N  L  R  E  K  I  E  P  L
caaaagcattatcatacaactgacaaagaatggtttggaaaatctgttttgctttcccat
 Q  K  H  Y  H  T  T  D  K  E  W  F  G  K  S  V  L  L  S  H
ctgcaccagctgaattccaaggcctcctgcacttgtcagtcgctgctgcttgatagaatg
 L  H  Q  L  N  S  K  A  S  C  T  C  Q  S  L  L  L  D  R  M
ttgaacatcactgaaacaattttgcaagacctgagagggaaagctgagaatgaagaaacg
 L  N  I  T  E  T  I  L  Q  D  L  R  G  K  A  E  N  E  E  T
aaaaccaggctaacagatgtaatgactgaggtgaaaatactgagacacaagtacagtgaa
 K  T  R  L  T  D  V  M  T  E  V  K  I  L  R  H  K  Y  S  E
gaacagaaagtatggagggagcttcaggacattcactcagtcgaggtgaaaaatggcaca
 E  Q  K  V  W  R  E  L  Q  D  I  H  S  V  E  V  K  N  G  T
aaccagaaaggagcactaaattcctttctcattttgtatgatctggcctactgaggaaaa
 N  Q  K  G  A  L  N  S  F  L  I  L  Y  D  L  A  Y  -
ccgagagagacaatcaccaagaatcatcttaatttctcttgctgtatagacagttattta
aattagtatttatttattccacttgactgttaaacaaataaaattacaaattttgcat
taaaaaaaaaaaaaaaaaaaaaaaa
```

(a) IFN-γ-1

```
atgactgcgcaaaacacaatggccttttctggggagtatgtttactgacttcaggatgg
 M  T  A  Q  N  T  M  A  F  F  W  G  V  C  L  L  T  S  G  W
atgacatacggcgaggccagcgtccctgagaacctggacaagagcattgatgagcttaaa
 M  T  Y  G  E  A  S  V  P  E  N  L  D  K  S  I  D  E  L  K
gcatactatataaaagatgatcatgagctacacaatgcacatcctgtcttcctacgggcc
 A  Y  Y  I  K  D  D  H  E  L  H  N  A  H  P  V  F  L  R  A
ctaaaagacttaaaggtgaatcttgaggaacctgagcagaatcttttgatgagcatcata
 L  K  D  L  K  V  N  L  E  E  P  E  Q  N  L  L  M  S  I  I
atggacacatacagtaggatatttactcgcatggagaatgatagtctggatgaagctaca
 M  D  T  Y  S  R  I  F  T  R  M  E  N  D  S  L  D  E  A  T
aaggaaagacttgcacatgttcaagagcatttgaaaaaactgaaagaaaactacttccca
 K  E  R  L  A  H  V  Q  E  H  L  K  K  L  K  E  N  Y  F  P
ggcaaaagtgcagagctcaagacatatgcagaaacactatgggcgatcaaggaagatgac
 G  K  S  A  E  L  K  T  Y  A  E  T  L  W  A  I  K  E  D  D
ccagtcatccagcgcaaagccttgtttgagctgaagcgtgtctacagagaagcaacacag
 P  V  I  Q  R  K  A  L  F  E  L  K  R  V  Y  R  E  A  T  Q
ttgagaaacctgaagaacaaagagcgcaggagacgacaagccaaaatcacaaaaaagcaa
 L  R  N  L  K  N  K  E  R  R  R  R  Q  A  K  I  T  K  K  Q
aagtcttag
 K  S  -
tagatcttgatcatcatttaaaagaatggtggaaaatcagtgttatttattctatttata
aagtttttgtacatatattgtgcacaacagacaattgtttttaactgacattatttattt
gttcttatttattttaaaaagcactgtaaaccacttgctagtaacatggcaaattttgta
aggtctgtgactgaataaaatcatttttttaagaaaaaaaaaaaaaaaaaaaa
```

(b) IFN-γ-2

Figure 7.1: Full cDNA sequence and deduced amino acid sequence of IFN-γ-1 gene (a) and IFN-γ-2 gene (b) of common carp. The predicted signal peptide is underlined, mRNA instability motifs (attta) are depicted in dark grey and polyadenylation signal (**aataaa**) is in bold. Primers to amplify the two genes are shown in italics in *light grey*.

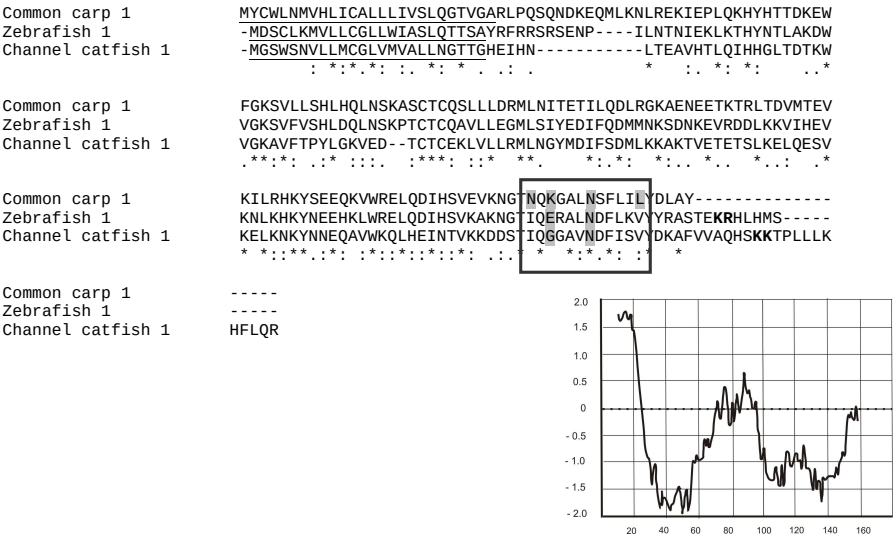
sequences. Therefore this sequence was designated common carp IFN-γ-1 (AM261214). The untranslated 3'region contains two instability motifs (ATTTA) and has a polyadenylation signal (AATAAA) 17 bp upstream of the poly[A] tail. The IFN-γ signature motif of higher vertebrates ([I/V]-Q-X-[K/Q]-A-X_2-E-[L/F]-X_2-[I/V]) is only partly matched and a nuclear localisation sequence (NLS) is absent (figure 7.2a). Consistent with these findings, the C-terminus of IFN-γ-1 is only slightly hydrophilic (figure 7.2a insert).

The second full-length sequence translated into a precursor protein of 182 amino acids with a putative 26 amino acid signal peptide (figure 7.1b). The precursor protein showed moderate sequence homology to other vertebrate IFN-γ sequences (20–50 %), but as the predicted protein showed 81 % amino acid identity compared to zebrafish IFN-γ-2 it was designated common carp IFN-γ-2 (AM168523). The gene contains five instability motifs in the untranslated 3'region and shows a polyadenylation signal (AATAAA) 13 bp upstream from the poly[A] tail. The IFN-γ signature motif is present and a nuclear localisation sequence of four contiguous basic amino acids is found at the C-terminus (RRRR) (figure 7.2b). A relatively high abundance of lysine and arginine results in a predicted strongly hydrophilic C-terminus (figure 7.2b insert).

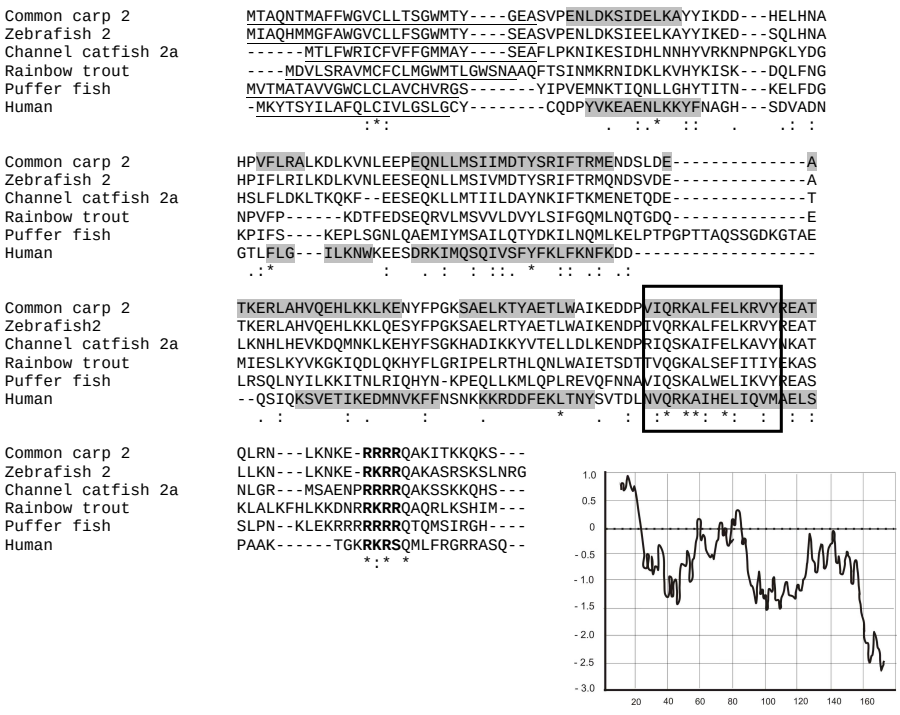
Phylogenetic analysis showed that the fish IFN-γ sequences cluster in a clade separate from the other vertebrate IFN-γ's. All the IFN-γ sequences form a monophyletic group that shares a common ancestor that differs from the type-I interferons. Within the fish clade a clear subdivision into two branches for IFN-γ-1 and IFN-γ-2 is seen (figure 7.3).

Identification of common carp T-bet and GATA3. Using homology cloning, partial T-bet (acc. AM944367) and GATA3 (acc. AM947129) sequences were detected in common carp. The partial T-bet sequence contains 326 amino acids, which is 54 and 61 % of the full complement of amino acids for crucian carp or human T-bet, respectively. Amino acid identity was > 90 % and > 95 % compared to crucian carp (acc. BAF73805) and zebrafish (acc. XP_001338262) and BLAST analysis yielded high similarity with T-box genes of other vertebrate species. The partial GATA3 sequence encodes 137 amino acids, which is 31 % of total amino acids in the zebrafish or human protein and amino acid identity is > 75 % compared to human (acc. P23771) and zebrafish (acc. NP_571286) and > 90 % compared to crucian carp (acc. BAF98873). BLAST analysis showed high similarity with GATA3 genes of other species.

Constitutive expression of IFN-γ-1 and IFN-γ-2 mRNA. The IFN-γ-1 was expressed at low level in head kidney, peripheral blood lymphocytes (PBL), spleen, kidney and gut, where mRNA expression was ten-fold lower compared to IFN-γ-2 expression. In thymus, liver, skin and gill expression was moderate and comparable to IFN-γ-2 expression. Stron-



(a) alignment IFN-γ-1



(b) alignment IFN-γ-2

Figure 7.2: Multiple alignment of deduced common carp IFN-γ-1 (a) and IFN-γ-2 (b) amino acid sequences with other vertebrate sequences. Multiple alignment was created using CLUSTALW software. (a) IFN-γ-1. Predicted signal peptides are underlined, and the IFN-γ signature motif is boxed, amino acid residues matching this signature motif ([I/V]-Q-X-[K/Q]-A-X_2-E-[L/F]-X_2-[I/V]) are depicted in grey. (b) IFN-γ-2. Predicted signal peptides are underlined, and the IFN-γ signature motif is boxed. Confirmed alpha helices in human IFN-γ and predicted alpha helices in common carp are depicted in grey. Nuclear localisation signal is shown in **bold**. Identical amino acids are indicated by *, and amino acids with high and low similarity are indicated as : and . respectively. Hydrophobicity plots for either gene are shown in insert and were generated using the Kyte-Doolittle method.

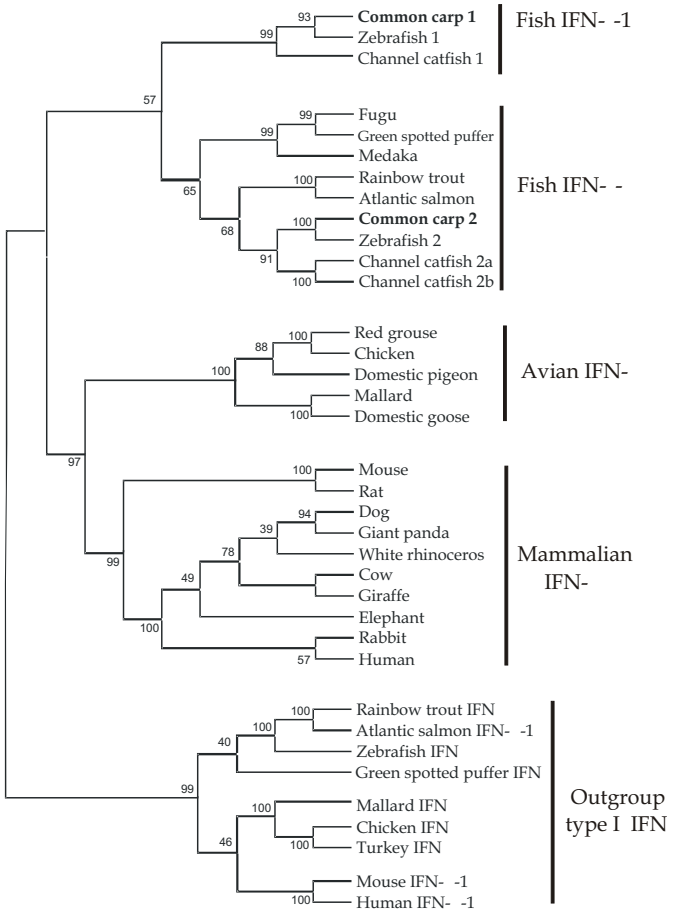


Figure 7.3: Phylogenetic tree, comparing the amino acid sequences of vertebrate interferon gamma genes. This tree was generated with MEGA version 3.1 software using the neighbour-joining method. Reliability of this tree was assessed by bootstrapping using 1000 bootstrap replications; values in percentage are indicated at branch nodes. Type I IFNs are used as outgroup. Common carp (*Cyprinus carpio*), IFN-γ-1; AM261214, IFN-γ-2; AM168523, Zebrafish (*Danio rerio*), IFN-γ-1; AB194272, IFN-γ-2; AB158361, IFN; AJ544822, Puffer fish (*Takifugu rubripes*) IFN-γ-2; AJ616216, Greenspotted puffer (*Tetraodon nigroviridis*), unnamed (IFN-γ); CAF95605, IFN; AJ544904, Rainbow trout (*Oncorhynchus mykiss*), IFN-γ; AJ616215, IFN; AY788890, Atlantic salmon (*Salmo salar*), IFN-γ; AY795563, IFN-α-1; AY216594, Channel catfish (*Ictalurus punctatus*), IFN-γ-1; DQ124249, IFN-γ-2a; DQ124250, IFN-γ-2b; DQ124251, Medaka (*Oryzias latipes*) pred. IFN-γ; ENSORLG00000020774, Domestic goose (*Anser anser*) IFN-γ; AY524421, Chicken (*Gallus gallus murghi*), IFN-γ; DQ906156, IFNα; DQ226092, Domestic pigeon (*Columba livia*) IFN-γ; DQ479967, Red grouse (*Lagopus lagopus scotia*) IFN-γ; DQ473434, Mallard (*Anas platyrhynchos*), IFN-γ; AAO13016, IFN; EF053034, Human (*Homo sapiens*), IFN-γ; P01579, IFN-α; NM_024013, Rat (*Rattus norvegicus*) IFN-γ; P01581, Mouse (*Mus musculus*), IFN-γ; EF423643, IFN-α1; NM_010502, Rabbit (*Oryctolagus cuniculus*) IFN-γ; AB010386, Dog (*Canis lupus familiaris*) IFN-γ; P42161, Cow (*Bos taurus*) IFN-γ; P07353, White Rhinoceros (*Ceratotherium simum*) IFN-γ; DQ305037, Giraffe (*Giraffa camelopardalis*) IFN-γ; EU000431, Elephant (*Elephas maximus*) IFN-γ; EU000432, Giant Panda (*Ailuropoda melanoleuca*) IFN-γ; DQ630727.

gest expression was seen in muscle, where IFN-γ-1 mRNA levels were five-fold higher than those for IFN-γ-2. IFN-γ-2 expression was seen in all typical immune organs such as head kidney, PBL, spleen, gut and thymus. Highest expression was found in gill and skin (without underlying muscle tissue) (figure 7.4).

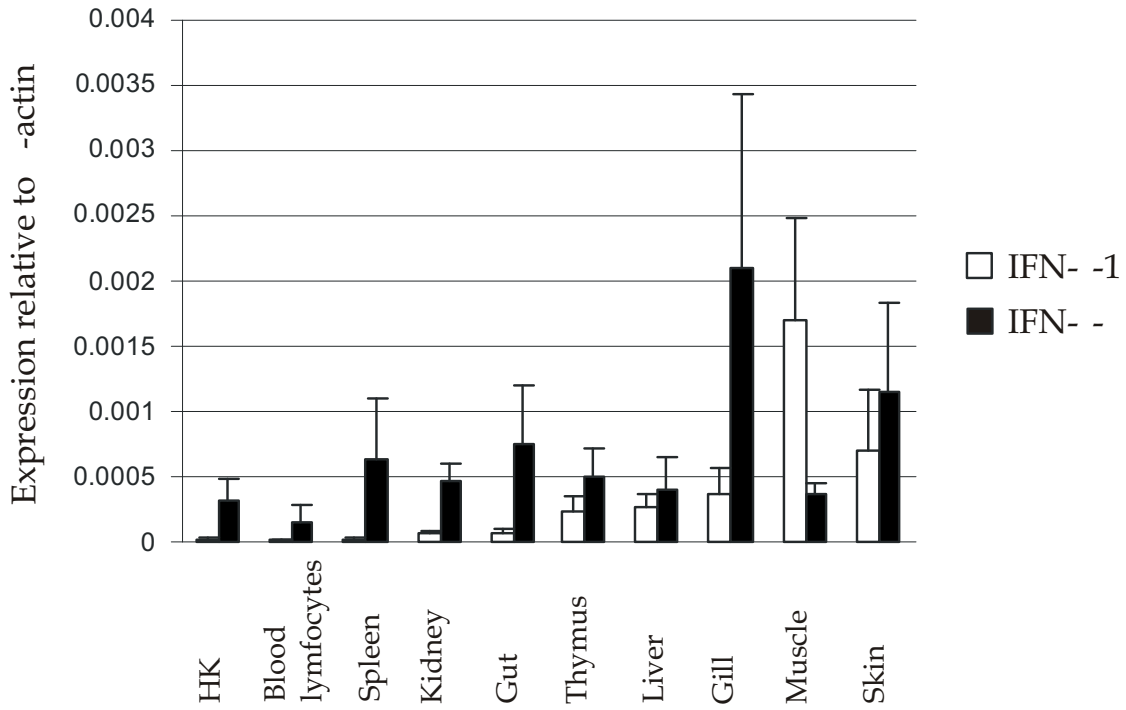


Figure 7.4: Constitutive IFN-γ expression. cDNA of different organs, or freshly isolated blood lymphocytes of four control fish, was used as template for quantitative real time PCR. Messenger RNA expression data is shown relative to the housekeeping gene β-actin. HK; head kidney.

In vitro induction of IFN-γ-1, IFN-γ-2 and T-bet mRNA expression. In a series of *in vitro* experiments, cells from different immune related organs were stimulated with 50 μg/ml LPS, 10 μg/ml PHA or 50 μg/ml poly I:C. To determine if the expression profile is consistent with a classical T-lymphocyte IFN-γ response, mRNA expression levels of IFN-γ-1, IFN-γ-2, GATA3, and T-bet, were determined simultaneously. GATA expression was not regulated after stimulation with LPS or PHA. B-cell stimulus LPS induced IFN-γ-1 expression in PBL and T-bet expression in head kidney phagocytes and PBL (figure 7.5a). T-cell stimulus PHA induced IFN-γ-2 expression in head kidney phagocytes, PBL and gut and gill leukocytes and induced T-bet expression in head kidney, PBL and gut leukocytes. IFN-γ-2 induction correlated with T-bet induction ($P < 0.01$) in head kidney and gut leukocytes. PHA also induced IFN-γ-1 expression in PBL and gut leukocytes (figure 7.5b). The viral ds RNA mimic Poly I:C did not reproducibly induce IFN-γ-1, IFN-γ-2 or either of the transcription

factors T-bet and GATA3 (figure 7.5c).

IgM$^-$ cells predominantly express IFN-γ-2 and IgM$^+$ cells express IFN-γ-1. IgM$^-$ (T-lymphocyte enriched) fractions showed a significant induction of IFN-γ-2 mRNA expression after PHA-stimulation and a small but significant increase of IFN-γ-1 expression after LPS-stimulation. Both the stimulation with LPS and with PHA increased T-bet expression in these fractions (figure 7.6a). IgM$^+$ (B-lymphocytes enriched) fractions showed significant induction of IFN-γ-1 expression upon stimulation with LPS, in concert with a very strong induction of T-bet expression (figure 7.6b). Poly I:C-stimulation did not reproducibly induce IFN-γ-1 or IFN-γ-2 expression (data not shown).

Mature thymocytes showed inducible IFN-γ-2 but not IFN-γ-1 mRNA expression. We used density separated mature (1.06 g/cm^3) and immature (1.07 g/cm^3) thymocyte cell fractions to determine if expression of either cytokine is maturation-dependent. Upon PHA-, but not LPS- or poly I:C- (data not shown) stimulation, IFN-γ-2 expression was upregulated in mature thymocytes. Immature thymocytes did not respond to any of the stimulants (figure 7.7a). Constitutive mRNA expression of IFN-γ-1 or IFN-γ-2 was similar in immature and mature cells (data not shown). However, constitutive expression of T-bet was significantly higher in mature cells compared to immature cells. Constitutive GATA3 expression did not differ between mature and immature thymocytes (figure 7.7b). As indirect IFN-γ induction via IL-12 may be anticipated, we determined expression of the latter gene. PHA-stimulation induced a 5-fold increase in p35 expression in either fraction and a 15-fold and 10-fold increase in p40 expression in mature and immature thymocytes respectively (data not shown).

IFN-γ-2 expression after parasite infection, not after zymosan-induced peritonitis. Zymosan-induced sterile peritonitis is characterised by influx of neutrophilic granulocytes and macrophages and the absence of lymphocytes in the peritoneal cavity (Chadzinska *et al.*, 2008). No significant differences in IFN-γ-1, IFN-γ-2, T-bet or GATA3 expression levels in head kidney (data not shown) or peritoneal leukocytes were found in infected compared to control fish at 96 hr (figure 7.8a). Parasite infection involves B-lymphocyte actions resulting in antibody production (Joerink *et al.*, 2007). Fish were sacrificed at peak parasitemia (3 weeks post-infection, data not shown) and IFN-γ-1 expression showed a slight but not significant increase in head kidney and gut cells. IFN-γ-2 mRNA levels were significantly upregulated in head kidney and spleen. Messenger RNA levels of both T-bet and GATA3 were increased in head kidney and in spleen (figure 7.8b).

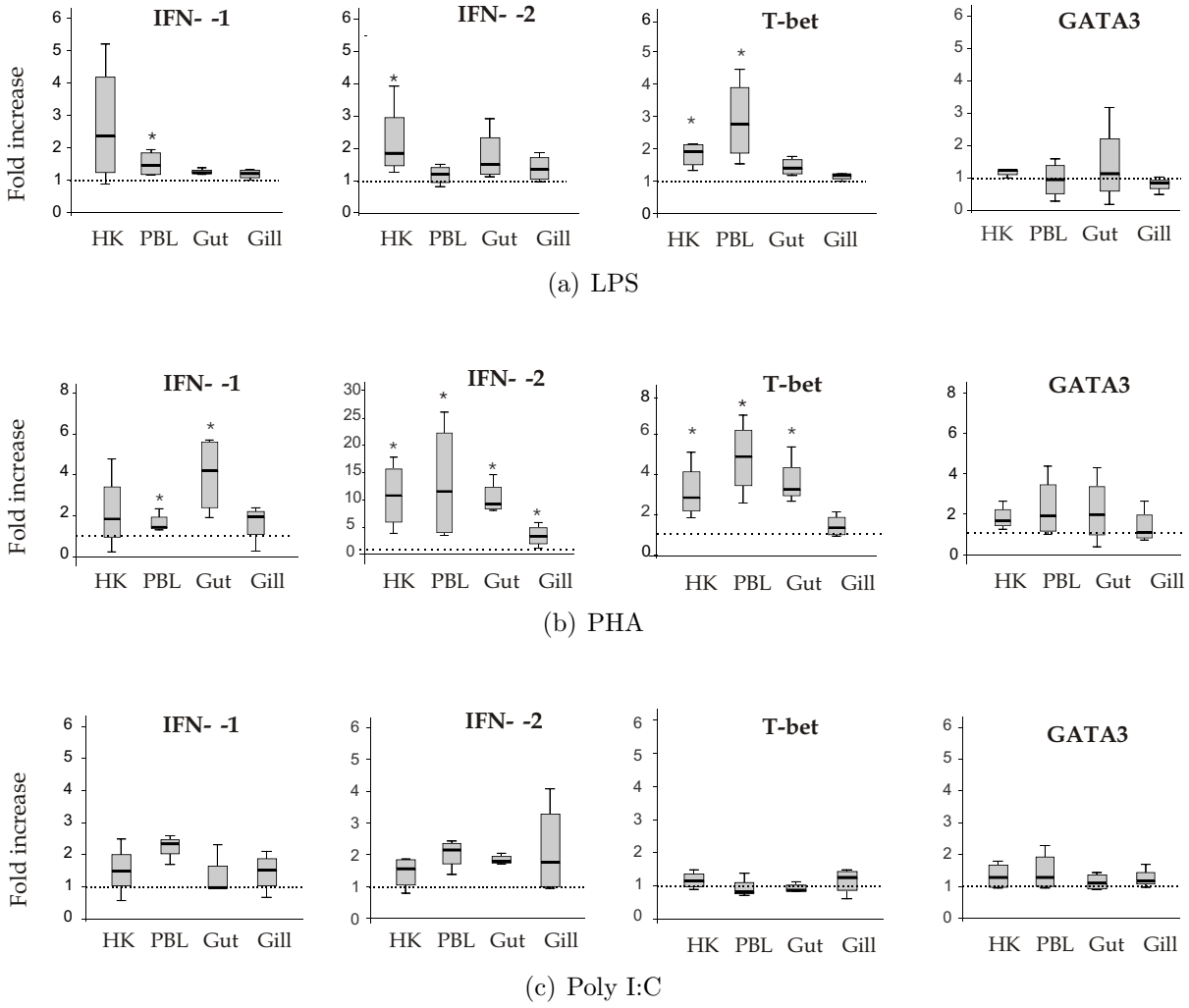
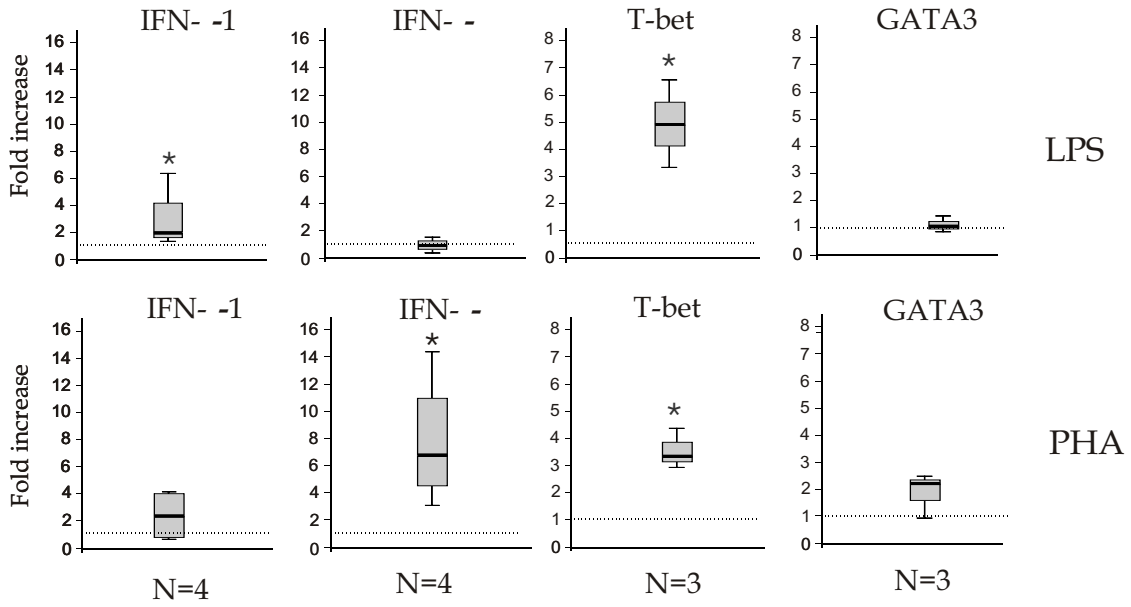
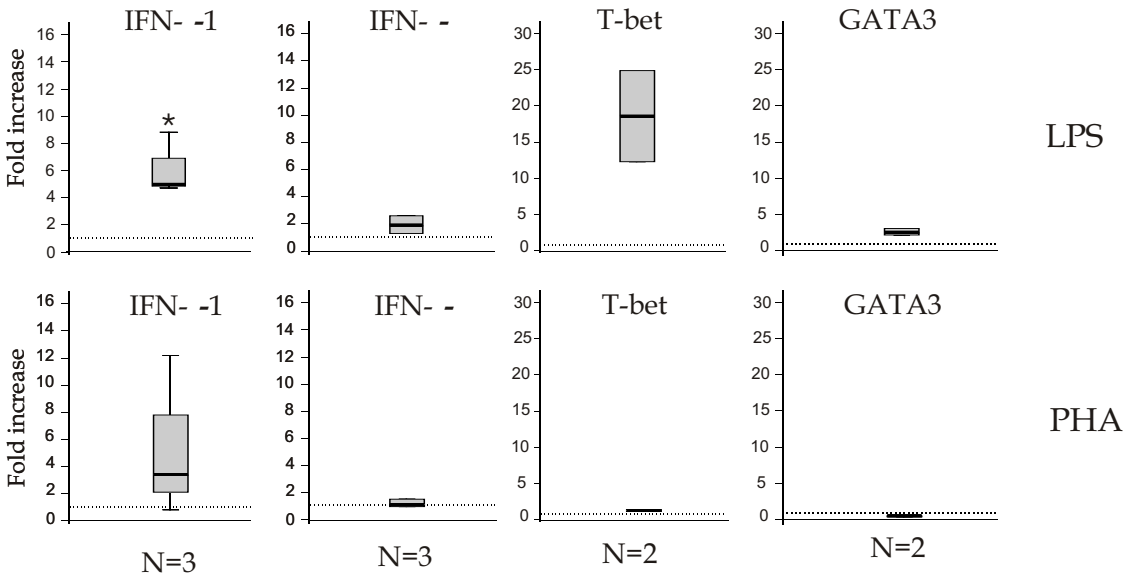


Figure 7.5: IFN-γ, T-bet and GATA3 induction in leukocyte cultures. Freshly isolated leukocytes were stimulated for 4 hr with 50 µg/ml LPS (a), 10 µg/ml PHA (b), or 50 µg/ml Poly I:C (c). Messenger RNA expression data of four control fish is shown as ×-fold increase compared to non-stimulated control cells, standardised for the housekeeping gene β-actin. HK; head kidney, PBL; peripheral blood leukocytes. Constitutive expression of control cells relative to the housekeeping gene β-actin; IFN-γ-1: head kidney phagocytes; $5.69 \times 10^{-6} \pm 1.07 \times 10^{-6}$, PBL; $1.10 \times 10^{-5} \pm 5.59 \times 10^{-6}$, gut leukocytes; $3.00 \times 10^{-4} \pm 2.49 \times 10^{-4}$, gill leukocytes; $8.45 \times 10^{-4} \pm 3.90 \times 10^{-4}$, IFN-γ-2: head kidney phagocytes; $1.04 \times 10^{-4} \pm 1.23 \times 10^{-5}$, PBL; $1.16 \times 10^{-4} \pm 3.34 \times 10^{-5}$, gut leukocytes; $1.06 \times 10^{-3} \pm 4.99 \times 10^{-5}$, gill leukocytes; $2.60 \times 10^{-3} \pm 1.08 \times 10^{-3}$.

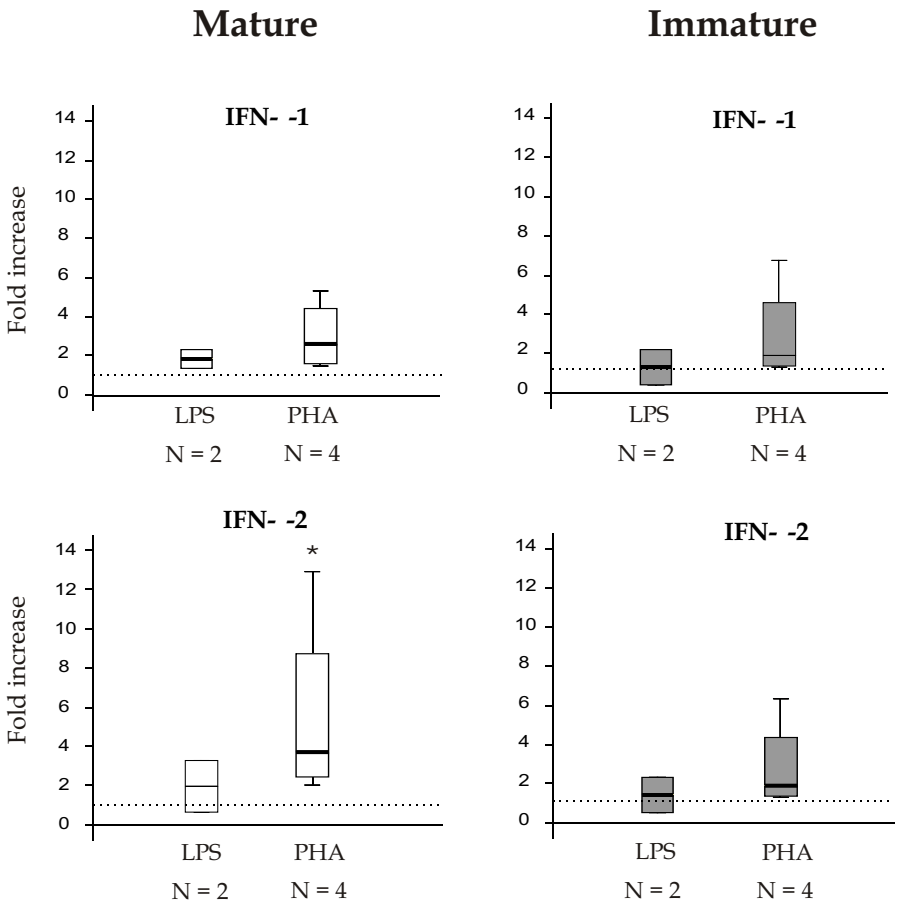


(a) IgM$^-$ (T-lymphocyte enriched)

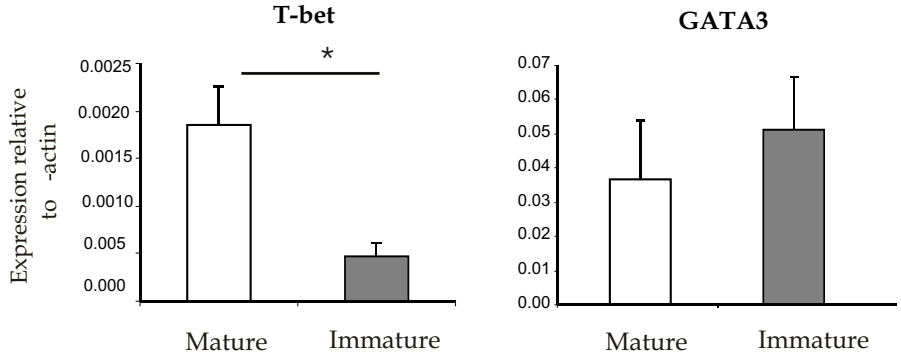


(b) IgM$^+$ (B-lymphocyte enriched)

Figure 7.6: IFN-γ, T-bet and GATA3 induction in MACS sorted blood lymphocytes. Freshly isolated leukocytes were sorted into IgM$^-$ (T-lymphocyte enriched) (a) and IgM$^+$ (B-lymphocyte enriched) (b) fractions and stimulated for 4 hr with 50 µg/ml LPS, or 10 µg/ml PHA. Messenger RNA expression data of four separate experiments is shown as ×-fold increase compared to non-stimulated control cells, standardised for the housekeeping gene β-actin. Constitutive expression of control cells relative to the housekeeping gene β-actin; IFN-γ-1: IgM$^-$ fraction; $5.75 \times 10^{-5} \pm 7.36 \times 10^{-5}$, IgM$^+$ fraction; $8.54 \times 10^{-5} \pm 6.26 \times 10^{-5}$, IFN-γ-2: IgM$^-$ fraction; $1.00 \times 10^{-3} \pm 1.15 \times 10^{-3}$, IgM$^+$ fraction; $1.33 \times 10^{-3} \pm 9.64 \times 10^{-4}$.

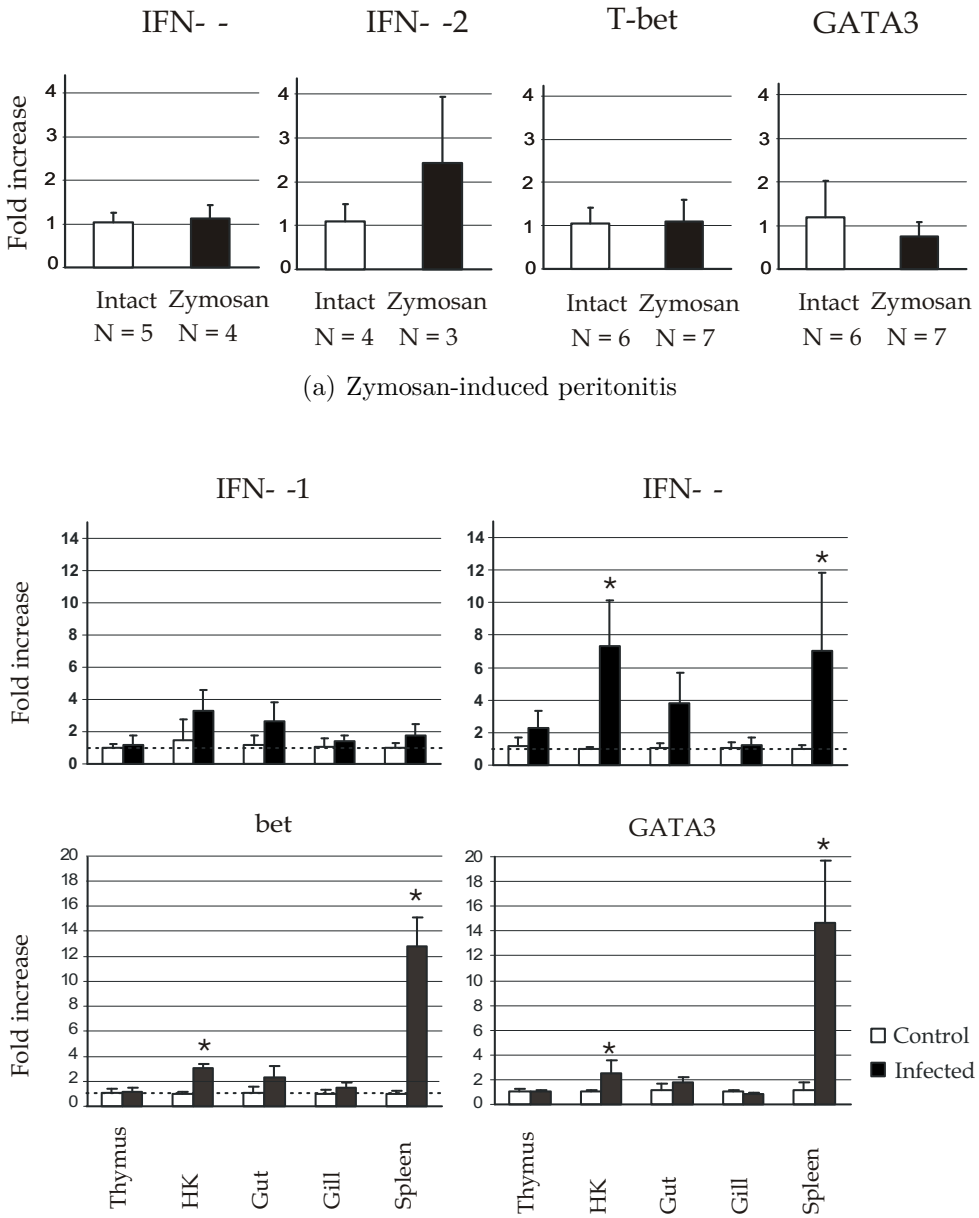


(a) IFN-γ expression



(b) Constitutive T-bet and GATA3 expression

Figure 7.7: IFN-γ induction in density separated thymocytes. Freshly isolated thymocytes were divided into mature and immature fractions by density separation and stimulated for 4 hr with 50 μg/ml LPS, or 10 μg/ml PHA. Messenger RNA expression data of four control fish is shown as fold increase compared to non-stimulated control cells, standardised for the housekeeping gene β-actin (a). Constitutive T-bet and GATA3 mRNA expression in mature and immature thymocyte fractions. Messenger RNA expression data is shown relative to the housekeeping gene β-actin and is plotted as average of four control fish, error bars indicate standard deviation (b). Constitutive expression of control cells relative to housekeeping gene β-actin; IFN-γ-1: mature thymocytes; $4.68\times10^{-5}\pm4.52\times10^{-5}$, immature thymocytes; $2.32\times10^{-5}\pm2.12\times10^{-5}$, IFN-γ-2: mature thymocytes; $1.15\times10^{-4}\pm9.98\times10^{-5}$, immature thymocytes; $6.21\times10^{-5}\pm7.43\times10^{-5}$.



(a) Zymosan-induced peritonitis

(b) *T. borreli infection*

Figure 7.8: IFN-γ, T-bet and GATA3 induction during peritonitis and parasite infection. 96 hr after injection, freshly isolated peritoneal leukocytes from intact and zymosan-injected animals were used as template for real time quantitative PCR. Messenger RNA expression data of four control and four experimental fish is shown as ×-fold increase compared to intact animals, standardised for the housekeeping gene 40S. Constitutive expression of control cells relative to housekeeping gene 40S; IFN-γ-1: $1.42 \times 10^{-3} \pm 3.00 \times 10^{-4}$, IFN-γ-2: $7.22 \times 10^{-3} \pm 2.14 \times 10^{-3}$ (a). Three weeks after parasite infection, freshly isolated tissues of four control and four infected fish were used as template for real time quantitative PCR. Messenger RNA expression data is shown as ×-fold increase compared to control fish, standardised for housekeeping gene β-actin. HK; head kidney Constitutive expression of control cells relative to the housekeeping gene β-actin; IFN-γ-1: thymus; $2.68 \times 10^{-4} \pm 6.25 \times 10^{-5}$, head kidney; $6.63 \times 10^{-5} \pm 5.80 \times 10^{-5}$, gut; $1.22 \times 10^{-4} \pm 6.09 \times 10^{-5}$, gill; $6.48 \times 10^{-4} \pm 2.26 \times 10^{-5}$, spleen; $1.55 \times 10^{-4} \pm 2.54 \times 10^{-4}$, IFN-γ-2: thymus; $9.02 \times 10^{-5} \pm 4.33 \times 10^{-5}$, head kidney; $4.21 \times 10^{-4} \pm 3.85 \times 10^{-5}$, gut; $4.89 \times 10^{-4} \pm 1.42 \times 10^{-4}$, gill; $3.85 \times 10^{-4} \pm 1.28 \times 10^{-4}$, spleen; $1.39 \times 10^{-4} \pm 1.52 \times 10^{-4}$. HK; head kidney, (b).

7.4 Discussion

IFN-γ and transcription factors T-bet and GATA3 play a crucial, well established role in T-cell development and differentiation in mammals, but little is known about the role of these genes in early vertebrate species. As fish have two different IFN-γ genes we used expression profiling to determine their roles in immune function and their share in teleostean T-lymphocyte function.

Genomic duplication is a now well-known phenomenon in teleostean fishes (Huising *et al.*, 2005, 2006c; Igawa *et al.*, 2006) and allows for subfunctionalisation. The duplication-degeneration-complementation (DCC) model as described by Force *et al.*, predicts that degenerate mutations are more likely to be preserved if ancestral functions are partioned (subfunctionalisation) rather than evolving into new functions (Force *et al.*, 1999). The conservation of both IFN-γ genes appears a result of this subfunctionalisation and is reflected by the structural differences and expression profiles of the resulting gene products. The marked differences between fish and vertebrate IFN-γ suggest that their divergence is an evolutionary old event. Likewise, the marked difference between fish IFN-γ-1 and IFN-γ-2 suggest that their divergence is an equally old event. Moreover, the presence of two IFN-γ genes is of wider occurrence among bony fishes, which suggests a duplication event prior to the teleostean split some 300 million years ago (Volff, 2005). As typical IFN-γ structural features are already present in common carp IFN-γ-2 and were retained in all vertebrate IFN-γ genes, we predict that functional characteristics from the common ancestor may have been retained. We set out to search for a genuine T-lymphocyte associated common carp IFN-γ-2.

Teleostean fish IFN-γ is widely expressed, which may reflect expression in multiple cell types, or expression of only a few cell types, (*e.g.* T-cells and NK-cells) that reside in epithelia. Common carp IFN-γ-2 is primarily expressed in T-lymphocyte associated tissues such as thymus, gill, gut and skin (Rombout *et al.*, 1998) but expression is also found in the phagocytic fraction of head kidney (primary site of erythropoiesis and functional equivalent of the mammalian bone marrow), and in IgM^+ fractions. Moreover, the T-cell stimulant PHA induced both T-bet and IFN-γ-2 expression *in vitro*. PHA in fish was also shown to induce expression of IL-2 (Bird *et al.*, 2005a) and NF45 (ILF2), a subunit of the nuclear factor of activated T-cells (NF-AT) (Lin *et al.*, 2006). Furthermore, indirect stimulation of IFN-γ expression by IL-12 is considered possible as ConA and PHA can induce the Il-12 subunits p35 and p40-1 in carp (Huising *et al.*, 2006c). Indeed, also in our *in vitro* assays both p35 and p40-1 were induced in thymocytes after PHA-stimulation.

Although constitutive IFN-γ-2 expression was found in IgM^+ (B-lymphocytes enriched) fractions, this expression was insensitive to LPS- or PHA-stimulation. Only the IgM^- (T-lymphocyte enriched) fraction showed induction of expression after PHA-stimulation. In accordance, in the IgM^- fraction we detected concomitant induction of T-bet, whereas this T-bet induction was not found in the IgM^+ fraction. Moreover, both immature and mature thymocytes constitutively expressed IFN-γ-2. However, only in mature thymocytes PHA-stimulation increased this expression. Interestingly, although T-bet expression after PHA-stimulation did not correlate with IFN-γ-2 expression, constitutive T-bet expression in mature thymocytes was significantly higher than in immature thymocytes. We hypothesise that acquirement of higher levels of T-bet expression reflects functional T-lymphocyte maturation, which enables responsiveness to immune stimuli. The apparent weak PHA responsiveness of T-bet in these cells implicates involvement of additional transcription factors for which on the basis of mammalian literature *e.g.* NFAT1 and AP1 are candidates (Lee *et al.*, 2004). These structural and *in vitro* expression data strongly support our hypothesis of a genuine T-lymphocyte associated IFN-γ-2 profile.

This hypothesis was moreover corroborated by two different *in vivo* model systems. In the first system, we used sterile zymosan-induced peritonitis which reflects a purely innate reaction during the early inflammatory response, characterised by influx of granulocytes and macrophages and absence of lymphocytes in the peritoneum (Chadzinska *et al.*, 2008). Therefore, if IFN-γ-2 is predominantly produced by T-lymphocytes, no induction of expression is predicted (which was found indeed). As a second, well characterised model, we used a parasite infection. The extracellular blood parasite *Trypanoplasma borreli* naturally is transmitted by blood sucking leeches and induces high nitric oxide (NO) production (Saeij *et al.*, 2002, 2000). Three weeks post-infection, when parasitemia and expression of pro-inflammatory cytokines reach peak levels (data not shown), significantly increased levels of IFN-γ-2 expression were found in head kidney and spleen, immune organs highly relevant to combat the high parasite load. Evidently, an effective anti-parasite defense requires a balanced differentiation between a type I and type II response. In mice, resistance to *Trypanosoma brucei* depends on the ability early during infection to produce IFN-γ, TNF-α but also NO (type I response) and the production of IL-4 and IL-10 (type II response) during the chronic phase. Imbalance induces tissue damage or a failure to control early pathogen replication (De Baetselier *et al.*, 2001). As in our *in vitro* experiments, increased IFN-γ-2 expression *in vivo* was accompanied by increased T-bet expression which suggests that IFN-γ-2 is under control of the T-bet transcription factor. Interestingly, GATA3 was also significantly increased in head kidney and spleen. This could reflect the onset of late

phase type II-like response, which is supported by the increase of anti-inflammatory cytokine IL-10 expression from three weeks onward to counteract the damaging effects of high oxygen radical production (M. Forlenza, Cell biology and Immunology Group, Wageningen University, personal communication).

Characterisation of the second interferon gene was more challenging as direct comparison to other vertebrate IFN-γ genes is difficult. Although IFN-γ-1 shares several features with other IFN-γ sequences such as a signal peptide, mRNA instability motifs and, in zebrafish, the gene structure of four exons (Igawa *et al.*, 2006), differences that are very likely to affect its (cytokine) function, are intriguing. The IFN-γ signature motif is only partly present; channel catfish and zebrafish sequences match the consensus sequence at five of the seven defined sites, whereas common carp only has three matches (Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006). Furthermore, the hydrophilic and basic C-terminus is absent. And finally, all IFN-γ-1 sequences lack a complete version of the NLS that is required for nuclear translocation and cytokine function. One might argue that the absence of an NLS implies that IFN-γ-1 is a pseudogene, or that it has evolved to a different function. However, the gene for common carp IFN-γ-1 is constitutively expressed in all organs tested. Moreover we show inducible expression, which suggests biological relevance. Irrefutable proof of cytokine-like function however awaits expression of a recombinant protein.

As in channel catfish, highest expression was found in muscle, which might indicate a non-immune related function (Milev-Milovanovic *et al.*, 2006). However, relatively high expression was found in carp thymus (as in catfish), gill (as in zebrafish) and skin, which does indicate an immune function (Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006). Interestingly, we found that LPS-stimulation was able to induce IFN-γ-1 expression in both IgM^+ (B-lymphocyte enriched) and IgM^- (T-lymphocyte enriched) fractions, although induction of expression was more pronounced in the IgM^+ fraction. Recently IFN-γ production by IL-12-stimulated murine B-cells, was found to depend on T-bet and the IFN-γ receptor, regardless of co-stimulation with anti-CD40, IL-18, or LPS (Harris *et al.*, 2005; Szabo *et al.*, 2000). In carp, LPS-stimulation strongly induced T-bet expression in the IgM^+ (B-lymphocyte enriched) fraction, whereas the the IgM^- fraction only showed a moderate increase of T-bet expression.

Moreover, as the IgM^- fraction will contain some IgM^+ cells, we cannot exclude that the moderate increase of IFN-γ-1 expression results from this contamination. To refute this, we investigated thymocyte cultures to determine possible IFN-γ-1 expression by T-lymphocytes. Neither mature nor immature thymocytes could be induced to increase IFN-γ-1 expression. NK cells as potential source of this cytokine is considered less likely as

we detect hardly any NK cells with the 5C6 antibody in the PBL fraction, and more importantly, NK cells do not respond to LPS-stimulation (Rombout *et al.*, 1998). In our *in vitro* assays head kidney phagocytes in some cases increased IFN-γ-1 expression after LPS-stimulation, although a lingering effect of B-lymphocytes could be present as phagocyte fractions that result from density separation always contain some lymphocytes (van Kemenade *et al.*, 1994). Indeed, in the zymosan-induced peritonitis model, where clearly the majority of the leukocytes present are phagocytes, we found no induction of IFN-γ-1 expression (Chadzinska *et al.*, 2008). Three weeks post-parasite infection, IFN-γ-1 was slightly, but not significantly increased in head kidney and spleen. At this time, following peak of parasitemia, antibody production will commence, resulting in complement-mediated lysis of the parasites (Joerink *et al.*, 2007). Besides NO mediated effects on the parasite as a result of IFN-γ-2 induced macrophage activation we hypothesise about a function in antibody production. The increase at three weeks post-infection of IFN-γ-1 in head kidney, which contains many antibody producing cells and in spleen, which is important in memory formation, likely reflects the onset of a second phase of antibody production, but this notion requires further research. We hypothesise that in common carp B- and T-lymphocyte associated IFN-γ functions have been divided between the two genes. IFN-γ-1 seems primarily a B-lymphocyte related cytokine, whereas IFN-γ-2 could not be induced in IgM^+ cells, but appears genuinely T-lymphocyte associated; an example of subfunctionalisation of genes within a single species.

Summarising, we found division of IFN-γ functions in two different genes. The IFN-γ-1 gene appears a B-lymphocyte cytokine, while the IFN-γ-2 shows widely accepted T-lymphocyte associated IFN-γ functions. However, despite their apparent differential expression profile and function both IFN-γ-1 and IFN-γ-2 appear to utilise similar intracellular pathways with a pronounced role for the transcription factor T-bet. Likely, this is a conserved feature from the common ancestor of the two IFN-γ genes that resulted from tandem gene duplication.

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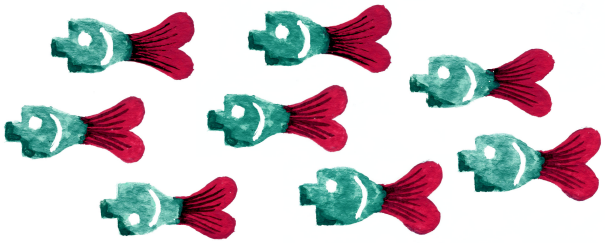

'Complicated things, everywhere, deserve a very special kind of explanation. We want to know how they came into existence and why they are so complicated.'

Richard Dawkins, The Blind Watchmaker

8

General discussion

Ellen H. Stolte

8.1 Aim of the thesis

The extant teleostean fishes represent the oldest and most successful vertebrates. Their success is exemplified by the estimated 23.000 species of fishes, compared to 14.000 species of amphibians, reptiles, birds, and mammals taken together. Fishes with their numerous adaptations offer an unique and interesting model for comparative studies and as they are among the earliest vertebrates they take an interesting position in evolution with important implications for phylogenetic analyses. They possess well-developed neuroendocrine and immune systems that depend on extensive bidirectional communication between these systems to guarantee allostasis. The maintenance of a balanced internal milieu is in fact based on dynamic equilibria of bidirectional processes (*e.g.* anabolism and catabolism, influxes and effluxes, *etc.*). Also the communication between the endocrine and the immune systems is bidirectional as these systems must coordinate their activities to ensure optimal stress coping and wound healing. An encounter with a pathogen may present an extrinsic stressor, which has to be communicated to the rest of the body. Conversely, the stress response has a dominant role in regulating immune cell numbers and (their) cytokine profiles and expression kinetics. An inflammatory response is necessary to overcome a bacterial infection, but a too strong inflammatory response can lead to destruction of host tissues.

Proceeding from this notion we investigated the role of the receptors for the stress steroid hormone cortisol in regulation of the immune response and the role of pro-inflammatory cytokines in regulation of the endocrine stress responses. We searched for the genes in carp that are involved in regulation of stress responses and immune response. Carp *Cyprinus carpio* is a very important species in aquaculture (as it accounts for 40 % of total aquaculture production of fish crustaceans and mollusks, (FAO, 2007) and its molecular biology is easily studied with the closely related zebrafish (*Danio rerio*) as reference (the zebrafish genome is completely known). Moreover, through our colleagues in Liverpool (A. Cossins and coworkers; (Cossins *et al.*, 2006; Gracey and Cossins, 2003)) we had access to a carp-specific microarray to study gene regulation in this fish.

8.2 Multiple receptors for cortisol

The evolution of multiple corticosteroid receptors and their signaling pathways in vertebrates was recently extensively reviewed (Ortlund *et al.*, 2007; Thornton *et al.*, 2003; Baker *et al.*, 2007; Bridgham *et al.*, 2006; Bury and Sturm, 2007; Prunet *et al.*, 2006) (**chapter 2**). The present consensus around the evolution of cortisol receptors is that an ancestral

corticosteroid receptor (AncCR) with less defined specificity for ligands transmitted cortisol and/or 11-deoxycorticosterone (DOC) signals in the ancestors of fishes; cortisol and DOC are the two main steroids produced in fishes (Bridgham *et al.*, 2006). All extant fishes lack, and, likely, fishes always lacked aldosterone synthase and thus do not produce aldosterone, the mineralocorticoid found in higher vertebrates. Duplication of the AncCR-gene over 450 million years ago led to separate genes for glucocorticoid (GR) and mineralocorticoid (MR) receptors (Baker *et al.*, 2007). It should be kept in mind that the appearance of the MR is the last event in the evolution of this group of transcription factors, which all originate from an ancestral estrogen receptor (Thornton *et al.*, 2003; Thornton, 2001); moreover, the appearance of the MR preceded the appearance of the ligand aldosterone. It is argued that the MR retained its ancestral phenotype (ability to bind both DOC, cortisol and aldosterone) and did not evolve as an aldosterone receptor (Ortlund *et al.*, 2007; Bridgham *et al.*, 2006). The capacity to synthesise aldosterone, indeed, evolved relatively recently in the lineage leading to tetrapods. As a result of modification of cytochrome P-450 11-β-hydroylase, this enzyme acquired the capacity to hydroxylate corticosterone allowing aldosterone synthesis (Bulow and Bernhardt, 2002). However, we (**chapter 3**) and others (Greenwood *et al.*, 2003; Sturm *et al.*, 2005) have shown that MR's may have physiologically relevant affinities and specificities for ligands that were to evolve after the appearance of the receptor (ligand exploitation theory; (Thornton, 2001)). The GR's ancestral sensitivity for aldosterone was most likely lost as a result of two crucial replacements, S106P and L111Q (Bridgham *et al.*, 2006).

A second major genomic duplication event took place in only teleostean fishes (not in tetrapods), and gave rise to further diversification of the glucocorticoid receptor family. Teleostean fishes, unlike all other vertebrates, have duplicate GR genes. This duplication occurred 450–300 million years ago, before the radiation of the actinopterigyans and after the divergence of the tetrapods from the fish lineage (Volff, 2005). Three possibilities arise after a genome duplication according to the duplication-degeneration-complementation (DDC) model as proposed by Force *et al.* (1999). First, non-functionalisation, *i.e.* one of the duplicates evolves as a pseudogene through degenerative mutations as redundancy reduces selective pressure on one gene copy. This is the fate of most duplicated genes; in the green-spotted puffer (*Tetraodon nigroviridis*) 85 % of paralogues were secondarily lost after the whole genome duplication (Brunet *et al.*, 2006). Second, neo-functionalisation, *i.e.* one copy acquires a new function, whereas the other copy keeps ancestral function. Third, subfunctionalisation, *i.e.* each copy loses part of the ancestral function and both copies are required to retain complete functionality (Lynch and Conery, 2000). This subfunctiona-

lisation can occur rapidly and is often accompanied by prolonged and substantial rates of neofunctionalisation (He and Zhang, 2005; Rastogi and Liberles, 2005). According to Brunet and coworkers, the whole genome duplication in fishes was followed by biased gene loss. The genes that were already under the strongest selective pressure were retained as duplicates after the genome duplication, which then resulted in enriched conditions for the genes in control of development, signaling pathways and complex behaviours (Brunet *et al.*, 2006). Indeed, Steinke and colleagues reported that the majority of genes that are suggested to have evolved under positive Darwinian selection are binding proteins, especially transcription factors and ATP-binding proteins .(Steinke *et al.*, 2006). The duplicated GR in teleostean fishes appears to support this concept. The duplicated GR genes in carp and other fishes require different concentrations of cortisol to initiate transcription of effector genes (Bury *et al.*, 2003; Greenwood *et al.*, 2003) (**chapter 3**), which is in line with neo-or subfunctionalisation to occur.

In mammals functionally different GRs result from polymorphisms or alternative spicing of a gene, an alternative mode to increase receptor variation and resulting corticosteroid sensitivity (DeRijk and de Kloet, 2008; DeRijk *et al.*, 2002). The human GR-β (hGR-β) shows alternative splicing of exon 9 leading to a shorter and different carboxy terminus (Bamberger *et al.*, 1995; Yudt *et al.*, 2003), which results in a nonfunctional ligand binding domain. This hGR-β is a dominant-negative inhibitor of hGR-α activity presumably via formation of inactive GR-α – GR-β heterodimers (Fruchter *et al.*, 2005; Lu and Cidlowski, 2004; Oakley *et al.*, 1999). A functional GR-β isoform has been described in only one fish species to date; the zebrafish (Schaaf *et al.*, 2008). Remarkably and surprisingly, this is the only fish species that has no duplicate GR genes. The zebrafish might compensate for the loss of a second GR gene by an alternative mechanism or regulation of glucocorticoid responsiveness, by means of this dominant negative GR (Schaaf *et al.*, 2008). Another GR described in humans and cotton top marmoset monkeys (*Saguinus oedipus*), GR-γ, shows alternative splicing in its DNA binding domain which adds an arginine (R) residue in the sequence. This R-insertion affects the capacity to initiate transcription (transactivation capacity) (Rivers *et al.*, 1999), but is not assumed to play an important physiological role in affecting glucocorticoid sensitivity (Stevens *et al.*, 2004a). Interestingly, the first teleostean GR genes that were described (rainbow trout (*Oncorhynchus mykiss*) and flounder(*Paralichthys olivaceus*)), showed a 9 amino acid insert in the DNA binding region at exactly the same location as the additional arginine in the human GR-γ (Ducouret *et al.*, 1995; Lethimonier *et al.*, 2002; Tokuda *et al.*, 1999). Subsequent studies on rainbow trout, carp and cichlids showed that the same gene is also expressed without this insert (Acerete

et al., 2007; Greenwood *et al.*, 2003; Takeo *et al.*, 1996; Vizzini *et al.*, 2007) (**chapter 3**). Both splice variants are either widely expressed, as in carp and Burton's Mouthbrooder (*Haplochromis burtoni*), or restricted to a single tissue (testis in rainbow trout); moreover, the transactivation capacities of the splice variant may vary (Greenwood *et al.*, 2003; Takeo *et al.*, 1996) (**chapter 4**). The functional differences in expression pattern and cortisol sensitivity support a scenario in which the duplicated GR genes are retained through a process of subfunctionalisations as proposed by the DCC model of Force *et al.* (1999).

8.3 Mineralocorticoid receptors?

As mentioned above, most genes after a gene/genomic duplication events may lose function and become a pseudogene (Brunet *et al.* (2006) and refs therein) Indeed, in carp one MR must have been lost during evolution as we cannot find evidence for a second MR-coding gene in this species, nor in genomic databases for zebrafish, green spotted puffer, puffer fish (*Fugu rubripes*) or rice fish (*Oryzias latipes*).

In mammals, corticosteroid regulation of the hydromineral balance is under control of the mineralocorticoid aldosterone, that exerts these actions via the MR. Corticosteroid receptors (CRs) are promiscuous for ligands, and this holds for the MR that 'sees' *in vivo* a variety of potential ligands. The precise interplay of signals with these receptors *in vivo* is still enigmatic. Intriguing is the enzymatic interference of 11-β-hydroxysteroid dehydrogenase-2 (11-β-HSD2), co-expressed with the MR in aldosterone targets, with cortisol signaling. The enzyme converts the far more abundant cortisol to inactive cortisone and, by doing so, gives aldosterone access to the MR (Funder, 2007). Aldosterone forms a cyclic 11,18 hemiacetal in solution and in that form escapes as substrate for the same enzyme (White, 1994). Fish, however, do not produce aldosterone as they lack aldosterone synthase (CYP11B2; (Jiang *et al.*, 1998); rather cortisol is thought to control osmoregulation (Marshall *et al.*, 2005; Metz *et al.*, 2003; Zhou *et al.*, 2003). Whether DOC should be considered a by-product of cortisol production (cortisol production in fish follows both the progesterone/DOC- and the 17-OH-progesterone/11-deoxycortisol pathway; (Li *et al.*, 2003) or is released as a proper ligand steering osmoregulation remains to be established. Little is known about regulation of cortisol levels by 11-β-HSD2 activity in fish, and the possible consequences of such enzyme activity for DOC activation of the MR. In fish levels of up to 10 nM DOC were published (Campbell *et al.*, 1980). The EC_{50} of DOC for MR transactivation in carp and rainbow trout, (0.25 nM and 0.1 nM respectively) (Sturm *et al.*, 2005) (**chapter 3**) certainly does not exclude a mineralocorticoid function for DOC in

fishes. Involvement of DOC and MR in osmoregulation in fish was suggested (McCormick *et al.*, 2005; Prunet *et al.*, 2006; Sturm *et al.*, 2005), but awaits further studies on plasma DOC profiles and physiological actions of the ligand.

The adverb 'mineralocorticoid' suggests a receptor binding ligand (aldosterone) steering osmoregulation and a mere function in the regulation of the hydromineral balance. Clearly, the terminology for hormones as well as receptors in this area is biased by functionality derived from the original mammalian studies. Our comparative physiological views now reveal that such extrapolation and generalisation based on studies on highly specialised species is disputable. With a EC_{50} of 4 nM, which is in between the EC_{50}s of the GR1(8–17 nM) and the GR2 (2 nM) the common carp MR could very well be a specific cortisol receptor (**chapter 3**). Similarly, in rainbow trout and Burton's mouthbrooder, MR cortisol sensitivity was either comparable or higher than GR cortisol sensitivity (Sturm *et al.*, 2005; Greenwood *et al.*, 2003). Interestingly, in mammals the MR binds the stress hormone cortisol with even higher affinity than the GR (Reul and de Kloet, 1985). Moreover, we showed that the common carp MR is expressed in non-osmoregulatory tissues (brain, pituitary gland, *etc.*), as was shown for mammals and rainbow trout. Apparently this is an old (at least 450 million years) design (**chapter 3**). The mammalian hippocampus exerts inhibitory control over HPA-axis activity: a dominant inhibition by MR activity is attenuated by GR activation (de Kloet *et al.*, 1998). MR expression in carp telencephalic pallial regions – where we should search for the early hippocampal equivalents in fishes (Meek and Nieuwenhuys, 1998) – and its downregulation by prolonged severe stress (**chapter 3**) is very reminiscent of the mammalian situation and point to a role for MR in early vertebrate stress regulation, a role that persisted then throughout mammalian evolution. It seems that a principal role in osmoregulation for the MR has only evolved with the ligand aldosterone in tetrapods, whereas a role in stress regulation appears ancestral. Reconsideration of the name MR based on the pivotal and original role in stress physiology and the ancestral function is warranted. Indeed, the definition given by stress physiologists after the discovery of two types of corticosteroid receptors rat brain seems appropriate. The Type 1 corticosteroid receptor refers to 'kidney mineralocorticoid receptor (MR)' and Type 2 is similar to the 'liver glucocorticoid receptor (GR)' (Reul *et al.*, 1987a; de Kloet *et al.*, 1986). We showed that the fish MR is primarily a stress receptor and might have an additional function in osmoregulation.

8.4 Function of multiple receptors in stress

The interesting picture arises that in fishes a single ligand (cortisol) may steer three different receptors (GR1, GR2 and MR); diversification therefore of receptors rather than of ligands. This concept together with the differential expression profiles and sensitivity for cortisol prompted our investigation towards differential functions for the MR and GR variants. The localisation of mRNA expression in components of the stress axis, in cells that produce corticotropin-releasing hormone (CRH) or adrenocorticotropic hormone (ACTH), and downregulated expression after prolonged stress implies that both duplicated GRs and MR are involved in stress axis regulation (figure 8.1). Together with the different transactivation capacities (concentrations of cortisol to initiate transcription of effector genes) (Bury *et al.*, 2003; Greenwood *et al.*, 2003) (**chapter 3**) suggests that subfunctionalisation of receptors has taken place (Force *et al.*, 1999). The 'sensitive' GR2 receptor is already activated at basal plasma cortisol levels, whereas the 'insensitive' GR1 requires stress levels of cortisol to become activated. MR sensitivity appears intermediate. The two variants (GR1a and GR1b) resulting from alternative splicing of the GR1 gene, did not show significant differences in constitutive expression or transactivation capacity and will therefore no longer be separately mentioned or discussed from here. However, it has to be reminded that although alternative splicing does not affect DNA binding and resulting activation of transcription, the 9 amino acid insert might affect interactions with co-activators and co-repressors (Kumar and Thompson, 2005). Fish have to control metabolic processes, regulate the hydromineral balance and maintain many other functions, all under control of one ligand. All these functions require, upon disturbance, differential regulation to realise allostasis. It is tempting to hypothesise that duplication of receptors as the result of a genome duplication enabled division of these functions under control of receptors with different cortisol sensitivity.

8.5 CRs in the immune system

Although it is generally assumed that stress inhibits the immune response, stress actually ensures a well-adjusted immune response, depending on the type, duration and intensity of the stressor. This differential regulation of the immune response could be mediated via different cortisol receptors. To test this hypothesis, we determined CR expression on the different cell types, and investigated CR expression in response to an immune stimulus.

Based on low constitutive mRNA expression in common carp leukocytes and mode-

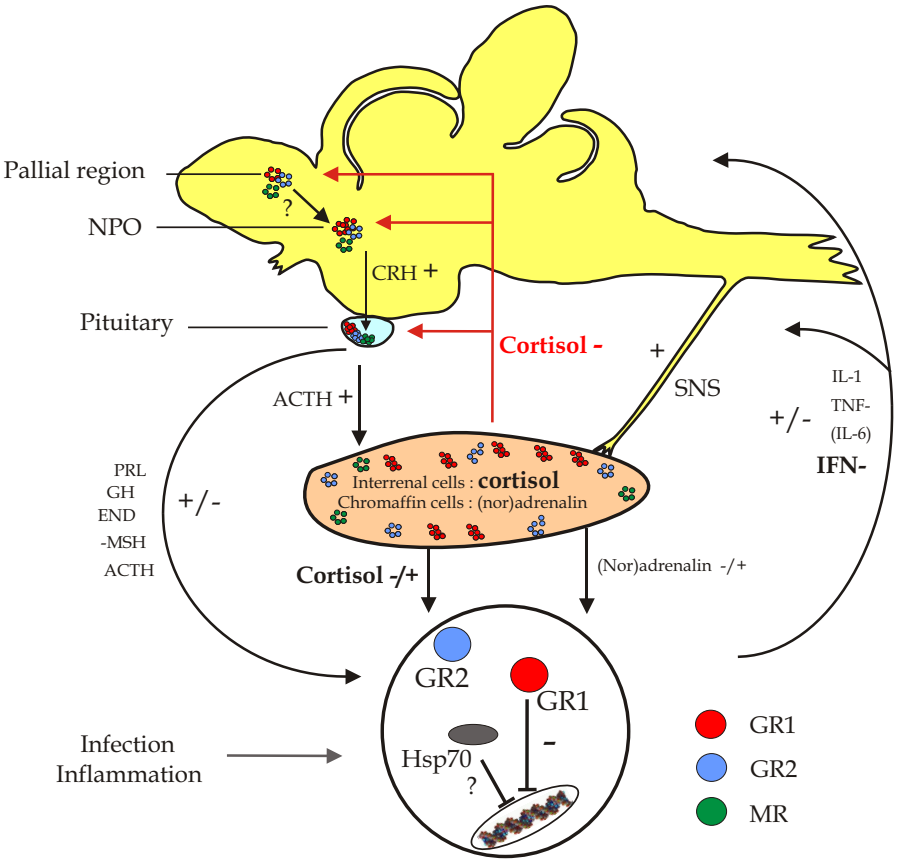

Figure 8.1: Proposed model for the neuroendocrine-immune interactions as described in this thesis. Pallial regions, comparable to the mammalian hippocampus, are hypothesised to signal to the nucleus pre-opticus (NPO) to release CRH. This CRH in turn induces ACTH release from the pituitary. Subsequently, ACTH induces cortisol release from the interrenal cells in the head kidney. Cortisol elicits its effects on metabolism, osmoregulation, stress axis activity and the immune system via three different receptors, GR1, GR2 and MR. GR2 is a sensitive receptor, which is already activated at low cortisol levels, whereas GR1 is only activated at high cortisol levels (such as found in stressed fish). MR has intermediate sensitivity for cortisol. The cortisol released into the circulation may inhibit stress hormone secretion (feedback) via the three receptors present in the pallial regions, NPO and pituitary. Chromaffin cells in the head kidney are under direct sympathetic control to release catecholamines. The immune cells from the fish originate from the head kidney and produce pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, and IFN-γ) to combat infection. Moreover these pro-inflammatory cytokines may affect hormone secretion in the pituitary and/or higher brain centres. Cortisol (via the corticosteroid receptors), pituitary hormones (PRL, GH, END, α-MSH and ACTH) and catecholamines can differentially affect cytokine production and immune cell function. Infection or inflammation increases expression levels of the 'stress' receptor GR1 and Hsp70. When activated by high levels of cortisol, GR1 (and Hsp70) inhibit expression of pro-inflammatory cytokines but receptor expression levels remain constant. If Hsp70 is required to allow GR1 function, or if Hsp70 can independently reduce pro-inflammatory cytokine expression is as yet unknown. Abbreviations: CRH; corticotropin-releasing hormone, ACTH; adrenocorticotropic hormone, PRL; prolactin, GH; growth hormone, END; endorphin, α-MSH; alpha melanocyte stimulating hormone, GR; glucocorticoid receptor, MR; mineralocorticoid receptor,SNS; sympathetic nervous system, IL-1β; interleukin-1 beta, TNF-α; tumor necrosis factor alpha, IL-6; interleukin-6, IFN-γ; interferon gamma.

rate transactivation capacity we concluded that GRs rather than MR conveyed the stress signal. *In vitro* stimulation with a bacterial cell wall product, viz. a *Escherichia coli* lipopolysaccharide (LPS) showed that the 'insensitive' or 'stress' receptor GR1 is predominantly increased during the early response (2 to 4 hr). Similarly the early phases (first 24 to 48 hr) of a zymosan-induced, sterile peritonitis increased GR1 expression (**chapter 5**). It is tempting to hypothesise that the transient increase in GR1 expression results in increased sensitivity for immune regulation by cortisol. We found that cortisol-treatment *in vitro* (100 nM, comparable to plasma levels in stressed fish), significantly inhibited LPS-induced upregulation of tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12) subunit p35 and IFN-γ-1 mRNA expression. Lower concentrations of cortisol, as measured in control fish did not significantly affect expression levels of the cytokines measured. Low concentrations of cortisol, although sufficient to activate GR2 are not enough to affect cytokine production. Therefore, we concluded that the 'stress' receptor GR1, is primarily involved in differentially regulating cytokine expression. Only after passing of the threshold for GR1 activation, pro-inflammatory cytokines levels are decreased in phagocytes (**chapter 4**). Moreover, in carp with zymosan-induced peritonitis, increased GR1 expression correlated with decreased pro-inflammatory cytokines IL-12 and IL-1β and increased anti-inflammatory cytokine IL-10 (Chadzinska *et al.*, 2008). Similar results were found in mammals, where glucocorticoids were found to inhibit the production of pro-inflammatory cytokines such as interleukin 12 (IL-12), interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) by antigen-presenting cells and T-helper (Th) 1 cells, but increased the production of anti-inflammatory cytokines IL-4, IL-10, and IL-13 by Th2 cells. Through this mechanism increased levels of glucocorticoids may cause a shift in the balance from the Th1-cellular immunity axis towards Th2-mediated humoral immunity, rather than generalised immunosuppression (Elenkov, 2004). This stress-induced immune modulation therefore seems an evolutionary conserved mechanism to prevent tissue damage as a result of a too strong pro-inflammatory response.

Interestingly, the glucocorticoid responses are shaped by molecular chaperones as has been recently described by Grad and Picard (2007). In mammals heat shock proteins (Hsp's) Hsp70 and Hsp90 are necessary to open the steroid binding cleft and enable binding of cortisol to the GR (Pratt and Toft, 2003) and a similar requirement is proposed in fish (Basu *et al.*, 2003). To investigate if heat shock proteins, as part of the ligand-receptor complex, were regulated during the glucocorticoid response, we investigated Hsp70 expression after stress- and immune stimulation. Heat shock proteins are chaperone proteins that assist protein folding (Kanelakis *et al.*, 2002), are co-factors for transcription (Grad and Picard,

2007) and are upregulated after cellular stress (stress that induces protein denaturation). Moreover, when cells are damaged, but not after apoptosis, heat shock proteins are released into the extracellular fluid and function as a 'danger signal' (Basu *et al.*, 2000). This signal is recognised by Toll-like receptors of the immune system and induces a strong pro-inflammatory response (Vega *et al.*, 2008). In carp Hsp70 expression profiles were comparable to those of GR1. Hsp70 shows very low constitutive expression and expression increases after an immune stimulus, *in vitro* or *in vivo*, but not after *in vitro* cortisol-treatment or restraint stress (**chapter 5**). Hsp70 has been shown to suppress LPS-induced production of pro-inflammatory cytokines and stimulate production of anti-inflammatory cytokines in mammals (Chen *et al.*, 2005; Shi *et al.*, 2006). Whether the increased Hsp70 expression in common carp is merely required to enable ligand binding to the GR1, or reflects the immune regulatory function of Hsp70 in its own right, remains to be determined (figure 8.1).

Besides differentially affecting the cytokine expression profile, differentially regulated expression of glucocorticoid receptors affects circulating immune cell populations. It has been shown before that physiologically realistic and low levels of cortisol induce apoptosis in carp B-lymphocytes, whereas T-lymphocytes are less sensitive to cortisol (Weyts *et al.*, 1998a) and neutrophilic granulocytes are actually saved from apoptosis by stress levels of cortisol (Weyts *et al.*, 1998b). This differential response may be explained by our observation that carp B-lymphocytes show a much higher level of the 'sensitive' GR2 receptor compared to neutrophilic granulocytes (**chapter 4**). A parallel is known for human neutrophils: in these cells the relative amounts of two GR splice variants determines glucocorticoid sensitivity. A high constitutive expression of the inhibitory GR-β in human neutrophils protects these cells for (sensitive) GR-α-mediated apoptosis. Moreover, variation in glucocorticoid responses between human monocytes and T- cells is suggested to result from different GR-β levels in these cells (Li *et al.*, 2006b). Moreover, transfection of cells with GR-β results in glucocorticoid insensitivity of these cells presumably via formation of inactive GR-α – GR-β heterodimers (Hauk *et al.*, 2002). Thus the apoptosis stimulating or inhibiting effects of cortisol depend on the immune cell type investigated, and appear a result of differential expression of particular glucocorticoid receptors. Differential glucocorticoid receptor sensitivity appears to have evolved independently in teleostean fishes and mammals.

8.6 Duplicate IFN-γ genes

IFN-γ had not been described in carp; it is a likely candidate for neuroendocrine modulation, also in fishes. Indeed, pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and IFN-γ act not only as signals in immune tissues and cells but also as neuromodulators and are key factors in central mediation of behavioral, neuroendocrine and neurochemical processes (Dantzer *et al.*, 2008; Kelley *et al.*, 2003). In mice, IFN-γ injection increases circulating prolactin levels; prolactin has opposite effects to those of cortisol (Cano *et al.*, 2005). Pro-inflammatory cytokine expression (IFN-γ) is inhibited by cortisol, but is increased after prolactin treatment (Dimitrov *et al.*, 2004). Also in fish, the immune modulatory actions of cortisol and prolactin are opposite as prolactin inhibits the decrease in proliferation blood leukocytes and the increase in apoptosis due to cortisol-treatment (Yada *et al.*, 2004). A possible neuroendocrine regulatory function for IFN-γ in production of cortisol and prolactin appears plausible but this awaits further direct experimental evidence.

To generate such evidence we first characterised the common carp IFN-γ as teleostean interferon gamma has not been extensively investigated. Common carp has also duplicate genes for IFN-γ that show differential expression profiles (**chapter 7**). This duplication is suggested not to result from a genomic duplication, but is in all likelihood the consequence of a tandem duplication (duplication of a part of a chromosome) as was suggested for zebrafish and channel catfish (*Ictalurus punctatus*) (Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006). The presence of two IFN-γ genes is of wider occurrence among bony fishes, which suggests a duplication event prior to the teleostean split some 300 million years ago (Volff, 2005). We now initiated to functionally characterise these duplicate genes. In common carp, duplicated IFN-γ genes are activated in response to different stimuli: IFN-γ-1 is largely expressed in LPS-activated B-lymphocytes, while IFN-γ-2 is expressed in T-lymphocytes following stimulation with the mitogen phytohaemagglutinin (PHA). This suggests partitioning of ancestral IFN-γ functions towards a cellular immunity driving IFN-γ-2 and a humoral immunity driving IFN-γ-1, in line with subfunctionalisation as described by the model of Force *et al.* (1999).

The Th1/Th2 paradigm in fish is still under debate. In mammals, after encountering an antigen, T-cells can adopt several effector functions. Th1 cells produce IFN-γ and IL-2 to effectively fight off intracellular pathogens, via cell mediated processes, whereas Th2 cells, by production of IL-4, IL-5 and IL-13 play a role in fighting multicellular parasitic infections through activation of humoral immunity. Differentiation of T helper cells into either the Th1 or the Th2 direction is primarily regulated by two transcription factors, GATA-3 and

T-bet (Glimcher *et al.*, 2004). Although the prominent Th1 pro-inflammatory cytokines IL-2, IL-12 and IFN-γ have been found in teleostean fishes, the key Th2 anti-inflammatory cytokine IL-4, although much sought after, has only been found in one species, the green spotted puffer (Li *et al.*, 2006a).

We hypothesise that IFN-γ-2 plays a Th1-like role by stimulating cell mediated immunity as indicated by the expression in T-lymphocytes. Indeed, rainbow trout recombinant IFN-γ(-2), was found to induce respiratory burst activity in head kidney macrophages (Zou *et al.*, 2005). In common carp, supernatant of PHA-stimulated thymocytes (presumably containing IFN-γ-2) induced expression of IL-12 and inducible nitric oxide synthase (*i*NOS) in head kidney phagocytes (unpublished observations). It is tempting to suggest that IFN-γ-1 has a Th2-like function in stimulating humoral immunity as it is primarily produced in B-lymphocytes in common carp, and was found to be expressed after treatment with B-cell stimulant LPS in common carp and in zebrafish (Igawa *et al.*, 2006). A preliminary study with recombinant IFN-γ-1 of common carp showed no effect on macrophage activity. Despite their apparent differential expression profile and possibly different function, both IFN-γ-1 and IFN-γ-2 appear to utilise similar intracellular pathways with a pronounced role for the transcription factor T-bet. This is likely a conserved trait from the ancestral IFN-γ. Although 'Th2 transcription factor' GATA3 is expressed in common carp, it was not found to be correlated with IFN-γ-1 expression. However, it was strongly increased in spleen after parasite infection, where greatly enlarged B-cell areas were detected and a significant increase in antibody levels was measured (Forlenza *et al.*, 2008b). Therefore an additional Th2-like cytokine under control of GATA3, involved in regulation of antibody production, such as IL-4, may be present. The duplication of IFN-γ genes appears to have resulted in differential functions for IFN-γ-1 and IFN-γ-2, steering humoral and cell mediated immune responses, respectively.

8.7 Evolutionary adaptations in the neuroendocrine and immune systems

As mentioned above, most duplicated genes, resulting from genome duplication become a non-functional pseudogene. However, as previously shown, this does not apply to our duplicated GR genes and duplicated IFN-γ genes as both duplicates have retained a partial function, required for maintaining allostasis. Endocrine regulation often addresses homeostatic or allostatic phenomena that are rather predictable and have remained basically comparable during the evolution of the vertebrates. For instance, sugar handling following

a meal in all vertebrates involves insulin or insulin-like molecules and glucagon as well as glucagon-related peptides and carbohydrate metabolism and energy flow in the body are under dominant glucocorticoid control by cortisol or corticosterone throughout the vertebrate lineage. In bone physiology in all vertebrates calcitriol, calcitonin and parathyroid hormone and/or parathyroid hormone related peptide are the key players. The receptors for all of these ligands are numerous and omnipresent in all vertebrates. Mutations in the genes coding for the signaling ligands and their receptors in the neuroendocrine system are more likely to be deleterious than beneficial for the species as the regulation concerns aspects of the organism proper. The presence then of duplicated glucocorticoid receptors, that appear to have a similar function but differentiate in sensitivity for the ligand, substantiate this notion. The ligands and receptors of the endocrine system thus have been 'optimised' over time.

One system that however really underwent a strong evolution in the vertebrate lineage is clearly the immune system, which reflects the rapidly and ever radiating pathogens that the immune system targets. On the one hand, variability and adaptability of the genes coding for the immune system continuously supplies the animal with selective and new ways to effectively combat pathogens. However, the same pathogens experience positive evolutionary pressure and are selected to evade the immune system, establishing a continuing arms raise. Diversification, reflected by radiation of cytokines and chemokines in fish (Huising *et al.*, 2006b; Lutfalla *et al.*, 2003; Nomiyama *et al.*, 2008), gives flexibility which is beneficial in dynamically changing environments and conflict situations. Indeed, the pleiotropy and redundancy displayed by cytokines and their receptors (albeit to a lesser extent) enables more variation, when functions are shared and covered by related cytokines. Additionally retention of both duplicates with partially different functions after gene/genome duplication, further increases this diversification. Interestingly, whereas it is generally assumed that higher vertebrates have a highly evolved and more elaborate immune system it is now found that fish have in fact more chemokines and perhaps more cytokines than mammals. Whereas in humans 44 CxC chemokines are reported, a recent analysis of the zebrafish genome showed more than a 100 chemokines (Nomiyama *et al.*, 2008). Moreover, teleosten fishes have several duplicated cytokine genes; *e.g.* IL-11 (Huising *et al.*, 2005), IL-12 (Huising *et al.*, 2006c), IFN-γ (Igawa *et al.*, 2006; Milev-Milovanovic *et al.*, 2006) and probably TNF-α (Grayfer *et al.*, 2008; Saeij *et al.*, 2003b) and IL-10 (Lutfalla *et al.*, 2003), which gives ample opportunity for diversification of function. Differentiation of B- and T-lymphocyte stimulatory functions of the duplicate IFN-γ genes as suggested by our characterisation in common carp, would corroborate this hypothesis. The diversification

that played an important role in the evolution of host defense against infections probably followed different pathways in tetrapods and fish.

8.8 Conclusion

In this thesis we showed that duplicated glucocorticoid receptors, with different sensitivities for physiological levels of cortisol as occur in rest and during stress, are pivotal in differential, cortisol mediated, regulation of the immune system. Immune stimuli rather than increased cortisol levels control GR expression in immune cells, which underlines their role not merely in stress but also as an integral part of the immune response. The differentially regulated expression of GR genes plays an important role in creating a balanced pro- and anti-inflammatory cytokine profile, as well as immune cell viability and thus in an effective immune response. This thesis illustrates the importance of extensive and effective bidirectional communication between the neuroendocrine and immune systems, that enabled the evolutionary success of the teleostean fishes.

'You can't possibly be a scientist if you mind people thinking that you're a fool.'

Douglas Adams, So long, and thanks for all the fish

Summary

In this thesis we investigated the involvement of the receptors for the stress hormone cortisol in stress and immune regulation. We set out to characterise the pro-inflammatory cytokine interferon gamma (IFN-γ). Furthermore, we used a genome wide screen (microarray) to search for additional genes that might be involved in regulation of the stress or the immune response.

In teleostean fishes cortisol can be bound by different receptors encoded by at least three different genes. An ancestral corticosteroid receptor (AncCR) is assumed to have been an effective receptor for cortisol in the ancestors of fishes. An early genomic duplication in the fish lineage, over 450 million years ago, led to separate glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) genes, both of which retained the ability to bind cortisol. A second major genomic duplication event took place only in teleostean fishes (not in other vertebrates), and gave rise to duplicate GR genes (GR1 and GR2). Even more variants developed as a result of alternative splicing of the GR1 gene which introduces a nine amino acid insert in the DNA-binding domain of GR1a, GR1b does not have this insert.

To investigate how one ligand can regulate many and very diverse functions using multiple receptors, we describe the expression of GR1 (a and b), GR2 and MR and their sensitivity for cortisol in **chapters 3 and 4**. The three receptors are expressed in tissues that make up the neuroendocrine stress-axis (brain, hypothalamus and pituitary) and in cells that produce corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). Decreased mRNA expression in brain after prolonged stress suggests an involvement in regulation of hypothalamo-pituitary-interrenal (HPI)-axis activity. In cells of the immune system MR expression is very low compared to GR expression and GR2 is preferentially expressed in lymphocytes. Transactivation assays shows that GR1 is a relatively 'insensitive' or 'stress' receptor, which can only become activated at stress levels of, whereas GR2 is a 'sensitive' receptor that will already be activated at basal levels of cortisol such as occur in non-stressed fish. MR sensitivity for cortisol is intermediate. We predict by tertiary protein modelling and confirmed by transfection assays, that the transactivation capacity of both

splice variants (GR1a and GR1b) is similar. Based on the very low expression level in immune cells and the moderate transactivation capacity of MR we concluded that GRs rather then the MR primarily convey stress signals to the immune system. Next, we determined the expression profile of the duplicated GR genes in the immune system in **chapters 4 and 5** to investigate the regulation of stress-induced immune modulation. Simultaneously we investigated the expression profile of (among others) heat shock protein 70 (Hsp70). This protein is required for binding of cortisol to the GR, but also has intrinsic immune modulatory functions, as it was shown to downregulate LPS-induced pro-inflammatory cytokine expression *in vitro* and *in vivo*. In head kidney phagocytes we found that only physiological stress levels of cortisol could reduce LPS-induced expression of pro-inflammatory cytokines, a response that appears mediated by the 'stress' receptor GR1. Moreover, we found that Hsp70 and GR1 (a and b) expression is increased after an immune stimulus *in vitro* and *in vivo*, whereas 24 hr restraint stress or 100 nM cortisol-treatment hardly increases Hsp70 and GR1 expression levels. This suggests that an immune stimulus rather than increased cortisol levels increases the sensitivity for glucocorticoid regulation and thereby of the cytokine profiles in immune cells.

To find additional genes involved in bidirectional neuroendocrine-immune communication we applied a genome wide screen of 9000 randomly picked cDNA clones. This has the advantage of an unprejudiced overview of regulated genes, but the sensitivity of the technique is limited. In **chapter 6** we describe a microarray experiment in which we compared mild acute stress, to prolonged severe immune stimulation. We show that an immune response after parasite infection appears tightly regulated and comparable between individuals, whereas a mild acute stressor allows for more variable gene expression profiles. We found LOC406744 of the DUF727 protein family and nephrosin as new interesting candidate genes that may be involved in neuroendocrine-immune communication.

The key pro-inflammatory cytokine IFN-γ, which is hypothesised to affect neurotransmitter and hormone release, had not been investigated in carp. In **chapter 7** we show that carp have duplicate IFN-γ genes that are expressed in immune cells. IFN-γ-2 shows structural and functional characteristics simlar to those in other vertebrate IFN-γ genes and appears to be involved in T-lymphocyte function, whereas IFN-γ-1 is expressed in stimulated B-lymphocytes. Currently recombinant proteins are being produced which will enable us to further elucidate the role of both IFN-γ gene products in the immune system as well as in mediating the neuroendocrine stress response.

Interestingly, as explained in **chapter 8**, both the glucocorticoid receptor and the IFN-γ genes are duplicated. The duplication-degeneration-complementation (DCC) model has

been proposed as an explanation for the high retention of duplicate genes in fishes. The hypothesis assumes that following gene duplication, the two gene copies degenerate over time by random mutation to perform complementary functions that jointly match that of the single ancestral gene, termed 'subfunctionalisation'. Indeed it appears that the duplicate GR genes have divided the general and 'stress-related' functions, reflected by their different sensitivity for cortisol. The duplicate IFN-γ genes appear to have divided B- and T-lymphocyte functions as targets suggested by their gene expression profiles upon selective stimulation.

An important conclusion of this thesis is that duplicated glucocorticoid receptors and heat shock proteins are an integral part of the immune system. Immune stimuli rather than increased cortisol levels control GR and Hsp70 expression in immune cells. The differentially regulated expression of GR genes is at the basis of a balanced pro- and anti-inflammatory cytokine profile, immune cell viability and thus at the basis of the success of the fishes. This thesis illustrates the importance of extensive and effective bidirectional communication between the neuroendocrine and immune systems, which are at the basis of the successful evolution of the vertebrates.

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'Als je maar een beetje dom en willekeurig aanrommelt en al je mislukkingen door iedereen vergeten worden, kom je toch heel slim over.'

Bas Haring, Kaas en de Evolutietheorie

Samenvatting

Het citaat van Bas Haring bovenaan deze pagina is een populaire vertaling van het **idee** dat tijdens de evolutie allerlei mogelijke veranderingen in planten en dieren optreden, maar dat alleen de meest gunstige veranderingen een lang leven beschoren zijn. Alle 'mislukkingen' zullen niet overleven, terwijl de 'slimsten' voor de volgende generatie zorgen. Hierdoor lijkt er een ontwerp of vooropgezet plan aan het evolutieproces ten grondslag te liggen dat zorgt voor steeds 'slimmere' planten en dieren. Het is belangrijk om te begrijpen dat dit slechts een illusie is en dat de 'slimmeren' alleen een betere kans op overleven hebben dan de 'mislukkingen'.

dit principe wordt 'survival of the fittest' genoemd en het mechanisme erachter 'natuurlijke selectie'

Tijdens de evolutie zijn er in vissen andere veranderingen opgetreden en gunstig gebleken dan in zoogdieren. Tijdens mijn onderzoek heb ik naar veranderingen in het stresssysteem en afweersysteem van vissen gekeken en de gevolgen hiervan geanalyseerd. Voor degenen die niet bekend zijn met het stresssysteem en het afweersysteem van vissen volgt hier een korte uitleg. Daarna volgt het doel van mijn onderzoek en een beknopte samenvatting van de resultaten.

Stress bij vissen

Het doel van stress is om het lichaam klaar te maken om te vechten of te vluchten. Stress zorgt bijvoorbeeld voor een verhoogde hartslag en meer energie voor de spieren. Als de hersenen een **stressor** registeren, wordt de hypofyse (hersenaanhangsel) geactiveerd. De hypofyse maakt een hormoon dat met het bloed vervoerd wordt naar de **kopnier**. De kopnier maakt vervolgens cortisol dat via het bloed naar alle cellen in het lichaam getransporteerd kan worden (zie figuur 1.2). Cortisol (stresssignaal) kan in een cel worden gebonden door een ontvanger. Deze ontvanger gaat vervolgens naar de kern van

externe stimulus zoals temperatuurverandering of het zien van een natuurlijke vijand

een orgaan dat zowel bloedcellen als stresshormonen maakt

de cel en zet hier processen in gang om het lichaam klaar te maken voor een adequate reactie op de stressor (zie figuur 8.1). Om een overreactie van het lichaam te voorkomen, zorgt cortisol er tevens voor dat de gevoeligheid van het lichaam voor stress afneemt (er treedt gewenning op).

Het afweersysteem van vissen

Het doel van het afweersysteem is om ziekteverwekkers zoals bacteriën, virussen en parasieten op te ruimen. Het afweersysteem van vissen bestaat daarvoor uit verschillende soorten cellen, die vrijwel allemaal vergelijkbaar zijn met de afweercellen in ons eigen lichaam. De afweercellen worden gemaakt in de kopnier en getransporteerd via het bloed zodat ze overal in het lichaam kunnen komen. Er zijn cellen die bacteriën of virusgeïnfecteerde cellen opeten – de fagocyterende cellen. Deze cellen kunnen bovendien stoffen produceren die dodelijk zijn voor virusgeïnfecteerde cellen of bacteriën. Daarnaast zijn er B-cellen die **antilichamen** maken, om de herkenning van bacteriën of virusgeïnfecteerde cellen door de fagocyterende cellen te vergemakkelijken. T-cellen, tenslotte, kunnen virusgeïnfecteerde of tumorcellen doden, waarna ze door fagocyterende cellen worden opgegeten. Al deze verschillende cellen communiceren met elkaar door middel van **cytokinen**. Het is belangrijk dat ziekteverwekkers zo snel mogelijk worden opgeruimd. Maar het is ook belangrijk dat het afweersysteem niet te sterk reageert. De signaalstoffen die vrijkomen om de ziekteverwekkers te doden kunnen namelijk ook schadelijk zijn voor het eigen lichaam. Er is dus een goede balans nodig om de ziekteverwekkers te verslaan zonder daarbij te veel schade op te lopen.

herkenningstekens die op bacteriën of virusgeïnfecteerde cellen gaan zitten

signaalstoffen die de cellen van het afweersysteem in beweging zetten

Hoe werken het stresssysteem en het afweersysteem samen?

Vissen staan net als wij bloot aan steeds wisselende omstandigheden. Om adequaat te kunnen reageren op veranderingen moeten alle cellen in het lichaam weten wat er gebeurt. Daarvoor zijn er verschillende communicatiesystemen. Via zenuwbanen die door het hele lichaam lopen, kan gecommuniceerd worden door middel van elektrische prikkels. Communicatie kan ook plaatsvinden

via signaalstoffen (bijvoorbeeld de hierboven genoemde cytokinen) die tussen cellen, of in het bloed worden afgescheiden. Deze signaalstoffen kunnen worden ontvangen door cellen met de juiste **ontvangers**. Bepaalde signaalstoffen van het stresssysteem (zoals bijvoorbeeld cortisol) kunnen worden ontvangen en begrepen door het afweersysteem en signaalstoffen van het afweersysteem worden op hun beurt begrepen door het stresssysteem. Interferon gamma is een voorbeeld uit de zoogdierenwereld van een signaalstof uit het afweersysteem die ook begrepen wordt door het stresssysteem. In hoeverre vissen en zoogdieren dezelfde signaalstoffen gebruiken in de communicatie tussen afweersysteem en stresssysteem moet nog verder uitgezocht worden.

ook wel receptoren genoemd

De vragen

Vissen kunnen stress ondervinden als ze met veel dieren in een relatief kleine bak zitten. Dit is een situatie die vaak voorkomt bij het kweken van vissen op grote schaal (intensieve visteelt). Omdat vissen een gezonde voedselbron zijn, wordt de viskweek een steeds belangrijkere manier om te kunnen blijven voorzien in de toenemende vraag naar dierlijke eiwitten. Stress kan de afweer tegen ziekten verminderen. Bovendien kunnen besmettelijke ziekten zich snel verspreiden als de dieren dicht op elkaar leven. Voor het welzijn en de gezondheid van vissen is het daarom belangrijk om te weten hoe het afweersysteem van de vis werkt en welke effecten stress heeft op de werking van dit afweersysteem. We willen ook weten of bepaalde stoffen uit het afweersysteem effect hebben op het stresssysteem. Met deze kennis kan in de toekomst misschien een methode ontwikkeld worden om het afweersysteem van de vis te versterken en daarmee de kans op ziekten te verkleinen of stress bij vissen te verminderen.

Belangrijke vragen binnen dit onderzoek zijn:

1. Welke ontvangers zijn er voor het stresssignaal cortisol?
2. Waar zitten de ontvangers en hoe gevoelig zijn ze?
3. Welke signaalstoffen en ontvangers zijn belangrijk voor vissen?
4. Welke functie heeft de signaalstof interferon-gamma in vissen?

Het onderzoek en de resultaten

Om de hierboven genoemde vragen te kunnen beantwoorden, hebben we gebruik gemaakt van de karper, aangezien dit een belangrijke vissoort is in de visteelt (40% van alle vissen die gekweekt worden voor consumptie zijn karpers). Bovendien maakt het formaat van de karper het mogelijk om bloed af te nemen en de signaalstoffen hierin te meten.

Welke ontvangers zijn er voor het stresssignaal? De voorouder van de huidige vissen (en zoogdieren) had een effectieve ontvanger voor cortisol. Deze ontvanger werd gemaakt volgens een instructie die in het erfelijk materiaal opgeslagen lag. Een stukje erfelijk materiaal met daarop een dergelijke instructie wordt een gen genoemd. Een **genoomduplicatie** in de afstammingslijn van alle gewervelde dieren, ongeveer 450 miljoen jaar geleden, leidde tot twee afzonderlijke kopieën van ieder gen. In de loop van de tijd echter veranderde de instructie die op deze genen was opgeslagen. In zoogdieren geeft één gen instructies voor een ontvanger van het cortisolsignaal en is dus belangrijk bij de reactie op stress en het regelen van de **glucosespiegel**, vandaar de naam glucocorticoid receptor (GR). De ontvanger die volgens de instructies op het andere gen wordt gemaakt, kan behalve cortisol ook het hormoon aldosteron binden en heeft een rol in het handhaven van de concentraties aan mineralen en reguleert daarmee de bloeddruk, vandaar de naam mineralocorticoid receptor (MR). Een tweede genoomduplicatie, ongeveer 300 miljoen jaar geleden, heeft alleen in de vissenafstammingslijn plaatsgevonden (en niet in andere gewervelde diersoorten) en leidde opnieuw tot verdubbeling van alle genen. Van de meeste genen verdween een kopie in de loop van de tijd doordat het gen door toevallige **mutaties** niet meer bruikbaar was. Wij vonden aanwijzingen dat dat ook het geval is voor de ontstane twee genen die instructies bevatten om de MR te maken. Maar de twee glucocorticoid receptor genen (GR1 en GR2) die ontstonden, bleven wel allebei bestaan. Vissen hebben dus in tegenstelling tot zoogdieren drie verschillende ontvangers voor het stresshormoon cortisol (GR1, GR2 en MR). Dezelfde boodschap kan dus door drie ontvangers opgevangen worden.

verdubbeling van al het erfelijke materiaal

bloedsuiker; energie voor de spieren

veranderingen in de instructies in het erfelijke materiaal

Waar zitten de ontvangers en hoe gevoelig zijn ze? Om te zien waar de ontvangers in het lichaam zitten, hebben we gekeken naar waar de instructies afgelezen worden. Op deze plaatsen is het dan waarschijnlijk dat ook de

ontvanger gemaakt wordt. We vonden dat de instructies overal in het lichaam afgelezen worden. Dat is ook wel logisch omdat alle delen van het lichaam als één geheel op de stressor moeten reageren. De instructies voor de ontvangers worden ook afgelezen in de hersendelen die er uiteindelijk voor zorgen dat cortisol geproduceerd wordt door de kopnier (zie pagina 211). Waarschijnlijk spelen de ontvangers hier een rol in het ongevoeliger maken van de hersenen voor de stressor om een overreactie van het lichaam te voorkomen.

We konden ook laten zien dat ene variant van de glucocorticoid receptor (GR1) een relatief 'ongevoelige' of 'stress'receptor is. Deze ontvanger is alleen actief bij hoge niveaus van cortisol in het bloed, zoals bij stress het geval is. De andere variant (GR2) is een 'gevoelige' receptor die al bij lage niveaus van cortisol in het bloed, zoals in rust al gemeten kon worden, actief is. De gevoeligheid van de derde ontvanger (MR) ligt tussen de waarden van de andere twee in.

Vervolgens hebben we het effect van stress op het afweersysteem onderzocht door te kijken of de **instructies** voor de ontvangers van het stresssignaal ook gebruikt worden in het afweersysteem. Stimulatie van het afweersysteem (bijvoorbeeld door een bacteriële infectie) bleek een sterker effect op het aflezen van de instructie te hebben dan stress. Vooral het aflezen van de instructies voor de stressreceptor, en daarmee waarschijnlijk de hoeveelheid van deze ontvanger, nam toe. Het lijkt er dus op dat het afweersysteem de hoeveelheid van de ontvangers van het stresssignaal en daarmee de gevoeligheid voor dit signaal (cortisol) kan reguleren. Dit lijkt een interessante terugkoppeling omdat we ook hebben gemeten dat cortisol de afgifte van signaalstofffen uit het afweersysteem kan verminderen. Een infectie zorgt ervoor dat er signaalstoffen worden gemaakt door het afweersysteem om de indringers op te ruimen. Tegelijkertijd maakt een infectie het afweersysteem gevoeliger voor cortisol, wat er op zijn beurt weer voor zorgt dat er niet teveel signaalstoffen worden gemaakt om schade aan het eigen lichaam te voorkomen.

iedere cel in het lichaam bevat hetzelfde erfelijke materiaal en dus zijn de instructies overal voorhanden

Welke signaalstoffen en ontvangers zijn belangrijk voor vissen? Om andere signaalstoffen of ontvangers te vinden die betrokken zouden kunnen zijn in de tweerichtingscommunicatie tussen het hormoon- en het afweersysteem gebruikten we de microarray techniek. Hiermee kan bepaald worden **welke instructies** op een bepaald tijdstip afgelezen worden. We ontdekten dat de

van het totaal aan instructies dat in het erfelijke materiaal is vastgelegd

reactie van het afweersysteem na een parasietinfectie strak geregeld is; dezelfde instructies worden in dezelfde mate afgelezen, of juist niet meer afgelezen in alle proefdieren. Milde stress (vastzitten in een leefnet) echter, lijkt vooral het aflezen van extra instructies te stimuleren, maar de mate waarin varieert tussen de proefdieren. We zagen dat een aantal interessante nieuwe instructies die betrokken kunnen zijn bij de communicatie werd afgelezen. Of, en zo ja hoe, deze instructies belangrijk zijn bij de communicatie moet nog verder worden uitgezocht.

Welke functie heeft de signaalstof interferon gamma in vissen? De signaalstof interferon gamma speelt een belangrijke rol in het afweersysteem in zoogdieren. Bovendien wordt gedacht dat interferon gamma betrokken is bij de afgifte van hormonen en **signaalstoffen in de hersenen**. Om te onderzoeken of dit ook bij vissen het geval kon zijn onderzochten we of interferon gamma ook in vissen aanwezig is en welke functie het heeft. Wij ontdekten dat karpers twee verschillende instructies hebben om deze signaalstof te maken (zoogdieren hebben er maar één). Deze instructies worden vooral afgelezen in cellen van het afweersysteem. De variant van de signaalstof die volgens de ene instructie wordt gemaakt, lijkt qua structuur en eigenschappen op de signaalstof van andere gewervelde dieren en speelt waarschijnlijk een rol in de functie van T-cellen. De alternatieve instructie wordt gebruikt in gestimuleerde B-cellen en levert een signaalstof die mogelijk een rol speelt in de aanmaak van antilichamen (zie pagina 212). Op dit moment zijn we bezig om deze signaalstoffen zelf na te maken zodat we kunnen onderzoeken welke rol ze spelen in het afweersysteem en in de afgifte van hormonen.

ook wel neurotransmitters genoemd

Hoe nu verder. . . ?

Wij hebben met ons onderzoek aangetoond dat vissen verschillende ontvangers voor het stresshormoon cortisol hebben en dat deze ontvangers verschillende functies kunnen vervullen. Bovendien hebben we aangetoond dat de ontvangers voor cortisol een integraal deel zijn van het afweersysteem. Stimulatie van het afweersysteem zorg ervoor dat er meer van de stressreceptor gemaakt wordt op bepaalde afweercellen. Dit zorgt voor een juiste balans in de afgifte van signaalstoffen door het afweersysteem. Dit is van belang voor het effectief bestrijden van een infectie terwijl tegelijkertijd de schade

aan het eigen lichaam beperkt blijft. De effecten van de communicatie van het stresssysteem met het afweersysteem onderstrepen het belang om deze twee systemen niet los van elkaar te zien. Vervolgonderzoek zal zich moeten richten op de stressgevoeligheid van de verschillende celtypen van het afweersysteem en op de rol die signaalstoffen uit het afweersysteem spelen in het stresssysteem.

'So long, and thanks for all the fish'

Douglas Adams, The hitchhiker's guide to the galaxy

Dankwoord

Het is nu echt af ...

De meeste mensen in mijn omgeving hebben er nooit aan getwijfeld dat het af zou komen, en/of er hard aan bij gedragen dat dit proefschrift afgerond werd, dank daarvoor!

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we kunnen veel van elkaar leren! De stoere mannen van dierfysiologie (en Daisy en Angela) bedankt voor de hulp bij experimenten en de (soms volstrekt politiek-incorrecte) humor. Ook de interesse van de mensen van celfysiologie was hartverwarmend.

Nic and Armin, thanks for your help! You made me feel very welcome in your lab and we got some beautiful results (shown in two papers) in only three weeks. It was great!

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Ellen Kranenbarg–Stolte

'...dat niet de geboorte, het huwelijk of de dood, maar de gastrulatie de 'werkelijk belangrijke gebeurtenis in uw leven' is.'

Lewis Wolpert, Het triomferende embryo

Curriculum vitae

Helena Hendrika Stolte werd op 31 december 1978 geboren in Espel in de gemeente Noordoostpolder. In 1997 behaalde zij het VWO diploma aan het Zuyderzee College in Emmeloord en in datzelfde jaar begon zij met de studie Geschiedenis aan de Rijksuniversiteit in Groningen. Na een half jaar bleek dat er te weinig mogelijkheden waren tot zelfstandig onderzoek en na het behalen van haar propedeuse werd besloten om met deze opleiding te stoppen. In 1998 begon zij (tot grote opluchting van haar ouders) met de opleiding Biologie aan de Landbouwuniversiteit in Wageningen. Tijdens haar eerste afstudeervak maakte ze kennis met de immunologie en de karpers bij de vakgroep Celbiologie en Immunologie in Wageningen. In dit afstudeervak onder leiding van Mark Huising en dr. Lidy van Kemenade werden verschillende CxC chemokines en hun receptoren gekarakteriseerd. Voor haar stage vertrok zij voor 7 maanden naar het Vancouver General Hospital in Canada. Binnen het Department of Infectious Diseases deed zij in het lab van prof. Chow onderzoek naar de effecten van regulatoire T-cellen op B-celactivatie, ditmaal in muizen. Terug in Nederland studeerde zij in juni 2003 met lof af. In dezelfde maand begon ze als assistent in opleiding (AIO) bij de vakgroep Celbiologie en Immunologie van de Wageningen Universiteit in samenwerking met de vakgroep Organismale Dierfysiologie van de Radboud Universiteit in Nijmegen. Onder leiding van dr. Lidy van Kemenade, prof. dr. Gert Flik en prof. dr. ir. Huub Savelkoul, werd het promotieonderzoek aan de bi-directionele communicatie tussen het neuro-endocrien- en het immuunsysteem in karpers uitgevoerd. De resultaten van het in dit proefschrift beschreven promotieonderzoek werden gepresenteerd tijdens diverse (inter)nationale congressen en werden gepubliceerd in diverse internationale vakbladen. Sinds juli 2008 is zij werkzaam in de groep van prof. dr. Linde Meyaard bij afdeling Immunologie van het Wilhelmina Kinderziekenhuis in Utrecht.

List of publications

Stolte, E.H., Chadzinska, M., Przybylska, D., Flik, G., Savelkoul, H.F.J. and Verburg-van Kemenade, B.M.L. (2008). The immune response differentially regulates Hsp70 and glucocorticoid receptor expression *in vitro* and *in vivo* in common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology*, submitted.

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'It's clearly a budget. It's got a lot of numbers in it.'

George W. Bush, May 5th, 2000

Professional training program

The table below summarises the professional training program as followed during the preparation of this thesis. The program was approved and supported by the research school WIAS (Wageningen Institute of Animal Sciences).

The basic package	year	credits[1]
WIAS introduction course	2003	1.5
Biology underpinning animal sciences: broaden your horizon	2003	1.5
Phylosophy of science and ethics	2005	1.5
Scientific exposure	year	credits
International conferences		
9th ISDCI[2] congress, St. Andrews, Scotland	2003	1.4
NVVI[3] jaarcongres, Noordwijkerhout, The Netherlands	2003	0.6
5th International symposium on fish endocrinology (ISFE), Castellon, Spain	2004	1.4
SEB[4] annual main meeting, Barcelona, Spain	2005	1.4
12th Benelux congress of zoology, Wageningen, The Netherlands	2005	0.8
NVVI jaarcongres, Noordwijkerhout, The Netherlands	2005	0.6
10th ISDCI congress, Charleston, USA	2006	1.4
NVVI jaarcongres, Lunteren, The Netherlands	2007	0.6
NVVI jaarcongres, Noordwijkerhout, The Netherlands	2007	0.6
seminars and workshops		
Symposium Bioinformatics at the Interface, Utrecht, The Netherlands	2003	0.3
Symposium 'From mouse to aquatic defence systems', Wageningen, The Netherlands	2003	0.1
WIAS science day, Wageningen, The Netherlands	2004	0.3
PhD retreat, Nijmegen, The Netherlands	2004	0.6
Neurobiology of the CRF neuropeptide family, Nijmegen, The Netherlands	2005	0.3
WIAS science day, Wageningen, The Netherlands	2005	0.3
WIAS science day, Wageningen, The Netherlands	2006	0.3
Symposium Endocrine and immune communication, Nijmegen, The Netherlands	2006	0.1
Seminar Macrophages and the immune system, Wageningen, The Netherlands	2006	0.1
Seminar Immune response to viruses, Wageningen, The Netherlands	2006	0.1
WIAS science day, Wageningen, The Netherlands	2007	0.3

[1] according to the ECTS (European Credit Transfer and Accumulation System), in which 1 credit represents a workload of 28 hours.
[2] International Society of Developmental and Comparative Immunology
[3] Nederlandse Vereniging voor Immunologen
[4] Society for Experimental Biology

Presentations		
Poster; NVVI, Noordwijkerhout, The Netherlands	2003	1.0
Oral; PhD retreat, Nijmegen, The Netherlands	2004	1.0
Poster; 5th International symposium on fish endocrinology (ISFE), Castellon, Spain	2004	1.0
Oral; Fish immunology workshop, Wageningen, The Netherlands	2005	1.0
Oral; SEB annual main meeting, Barcelona, Spain	2005	1.0
Oral; 12th Benelux congress of Zoology, Wageningen, The Netherlands	2005	1.0
Oral; Fish Immunology Workshop, Wageningen, The Netherlands	2006	1.0
Oral; 10th ISDCI congress, Charleston, USA	2006	1.0
Poster; 23rd Conference of European comparative endocrinologists, Manchester, UK	2006	1.0
Oral; WIAS science day, Wageningen, The Netherlands	2007	1.0
Oral; Fish immunology workshop, Wageningen, The Netherlands	2007	1.0
Poster; NVVI, Noordwijkerhout, The Netherlands	2007	1.0
In-depth studies	year	credits
Disciplinary courses		
Fish immunology workshop, Wageningen, The Netherlands	2003	1.4
Introduction to bio-informatics (summer school), Nijmegen, The Netherlands	2003	2.8
qPCR seminar experimental design and data treatment, Nijmegen, The Netherlands	2003	0.1
Post graduate course immunology, Utrecht, The Netherlands	2004	1.4
Advanced statistics courses		
Practical statistics for microarray data, Utrecht, The Netherlands	2004	0.6
Design of animal experiments, Wageningen, The Netherlands	2005	1.0
Statutory courses		
Use of laboratory animals (artikel 9), Utrecht, The Netherlands	2001	
Laboratory use of isotopes (radiation expert 5B), Wageningen, The Netherlands	2004	1.4
Professional Skills Support Courses		
Techniques for scientific writing, Wageningen, The Netherlands	2004	1.1
Supervising MSc theses, Wageningen, The Netherlands	2004	0.8
Research skills training	year	credits
External training period		
University of Liverpool, performing micro-arrays, Liverpool, UK	2004	1.5
University of Liverpool, data analysis micro-arrays, Liverpool, UK	2005	0.5
Kings college London, performing transactivation assays, London, UK	2006	1.5
Didactic skills training	year	credits
Assisting laboratory exercises		
Fish Immunology Workshop, Wageningen, The Netherlands	2003	0.1
Fish Immunology Workshop, Wageningen, The Netherlands	2004	0.1
BSc course cell biology I, Wageningen, The Netherlands	2004	1.5
BSc course cell biology I, Wageningen, The Netherlands	2005	1.5
Fish Immunology Workshop, Wageningen, The Netherlands	2005	0.1
Fish Immunology Workshop, Wageningen, The Netherlands	2006	0.1
Fish Immunology Workshop, Wageningen, The Netherlands	2007	0.1
Supervising MSc theses		
Sandra Janssen – Expression of GRs and MRs in common carp	2005	2.0
Maria Jęsiak – Glucocorticoid receptors in common carp	2006	2.0

Annemarie Hendriks – Finding interferon-γ in common carp	2006	2.0
Greetje Castelijn – Regulation and expression of IFN-γ genes in common carp	2007	2.0
Management skills training	year	credits
Organisation of seminars		
WIAS science day	2005	1.0
WIAS science day	2006	1.0
Membership of boards and committees		
WIAS-associated PhD student council secretary (Jan 2004 - Jan 2005)	2004	3.0
WIAS-associated PhD student council chair (Feb 2005 - March 2006)	2005	4.0
Total professional training		62.7

Omslag: De omslag verbeeldt de volgende gedachten bij de inhoud van dit proefschrift. ‘Altijd haast. Altijd stress. En allemaal hebben ze een belangrijke boodschap. Soms een simpele, soms een meer ingewikkelde. Maar alles moet zo snel mogelijk afgeleverd worden. Nog even haasten. Nog even stressen. Het is maar goed dat ze altijd terecht kunnen. Dag en nacht. Vroeg en laat. En op de plaats van bestemming staan drie ontvangers er helemaal klaar voor. Dat is letterlijk van levensbelang.’

Omslagontwerp: Jan Kranenbarg