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ADAPTATION PHYSIOLOGY
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EARLY DEVELOPMENT
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ABSTRACT – This paper reviews aspects of stress physiology of (young) fishes and the perspectives of this knowledge for aquaculture practices and ecophysiology. We will describe our present ideas about endocrine stress axes in fish. Particular attention will be paid to new insights in the bi-directional communication between the neuro-endocrine stress axis and the immune system and the potential modulation of stress axis activity by the immune system as well as to stressful experiences during early development, with a focus on waterborn copper as a modulator of stress axis activity.

PHYSIOLOGIE DE L'ADAPTATION
AXE DU STRESS
DÉVELOPPEMENT PRÉCOCE
IMMUNOLOGIE
CUIVRE

RÉSUMÉ – Une synthèse des aspects de la physiologie du stress chez les jeunes Poissons et des perspectives liées à ces connaissances en aquaculture et en écophysiologie est dressée. Nous présentons nos idées actuelles au sujet du stress endocrinien chez les Poissons. Une attention particulière est accordée aux nouveaux aperçus en matière de communication bidirectionnelle entre l'axe du stress neuro-endocrinien et le système immunitaire, à la modulation potentielle de l'activité de l'axe du stress par le système immunitaire, ainsi qu'aux expériences inductrices de stress au cours du développement précoce, avec notamment l'action du cuivre véhiculé par l'eau en tant que régulateur de l'activité de l'axe du stress.

INTRODUCTION

Our insight in the endocrinology of (stress in) fish is progressing rapidly due to modern molecular biological technologies with ever increasing specificity and sensitivity. The specificity requirement is particularly crucial to fish research considering the phenomenal number of species and their genetic variability. Recent estimates for the number of species of fish come to 35.000 (T. Iwamoto, California Academy of Sciences, pers comm). As a consequence, only a very limited number of species has been studied and thus our knowledge concerning fish endocrinology can only be fragmentary. More important, however, is the notion that an essentially unlimited wealth of adaptation strategies is found within this largest group of the vertebrates. Fish are vertebrates and demonstrate homeostasis of their "milieu intérieur", which differs only in details from the situation as we encounter in terrestrial vertebrates and that we often consider the standard. Fish do so essentially and independently of their habitat. It is relevant to realise oneself that fish live in an aqueous environment (there are of course exceptions found in e.g. lungfishes, mudskippers and annual fish). Living in water imposes an imminent threat of disturbance of homeostasis of the fish, as the animal is in a very close contact with this environment via gills. In gills an enormous surface is exposed to the water. Gills are in most fishes covered by a delicate and fragile epithelium designed for gas exchange; in addition, the epithelium of fish gills harbours the so-called chloride cells (after their initially determined function of chloride extrusion in seawater fish), ion transporting cells (further called ionocytes) that govern ion exchange phenomena and ion balance of the fish. Key in the functioning of these cells is an abundance of ion-pumps (H^+-ATPase, Na^+, K^+-ATPase, Ca^{2+}-ATPase, Na^+/Ca^{2+}-exchange, Na^+, K^+, Cl^-- and Na^+, Cl^--cotransporters etc.) that are associated with an elaborate(d) basolateral plasma membrane compartment; of all the transporters the H^+-ATPase and Na^+, K^+-ATPase are nowadays considered to provide the primary driving forces

for the multiple ion movements seen (Flik *et al.* 1995). As ion transport processes related to gas exchange are linked to those meant to guarantee ion balance it follows that ion strength, ion species (e.g. Na^+, K^+, Cl^-, H^+, HCO_3^-), gas content (O_2, CO_2) as well as physical conditions (e.g. absolute pressure with its consequences for partial gas pressures, water temperature, mixing zones) affect a multitude of interlinked physiological processes. Another important consequence of the thin, non-keratinising lining of the branchial apparatus is the inherent risk of invading organisms (uni- and multicellular parasites) and antigens (when lipophilic or in the event of epithelial damage), for which fish had to develop a strong and dependable innate as well as acquired immunity to successfully exploit and explore all the aquatic habitats where we now find fish. As we now know, the immune system and the neuroendocrine system share signals and receptors and communicate with one another (Verburg-van Kemenenade *et al.* 2001, Weyts *et al.* 1999) and thus understanding stress physiology requires insights in both systems and their signals. In very young fish the development of the immune system is assumed to follow (in time) that of the neuroendocrine system. In carp thymus and headkidney (albeit without lymphoid cells) as recognizable structures are detected no earlier than 3 days post fertilisation and lymphoid cell do not appear in carp headkidney but after two weeks of development (Romano *et al.* 1997). It would seem then that carp larval stages may provide natural models of fish with a certain time window to study the functioning of the neuroendocrine system independently of signals from the immune system. However, molecular biological approaches (Dr Verburg-Van Kemenade, pers comm) have allowed detection of immune parameters much earlier and independently of the existence of the organs proper as seen in adults (thymus and headkidney; thus cellular components later found in these organs may be around and cover the functions associated with these organs in later stages). The carp then, with its rapid development (hatching between 48 and 72 hours post-fertilization at 24° C) may be a less suitable model to test this hypothesis, but a multitude of fish species with slower developmental rate are at hand and we will focus the research in our laboratory on this topic in the near future.

Indeed, fishes inhabit every thinkable (aquatic) niche on earth: ion-rich – hyper-ionic compared to the blood plasma – waters like seawater, concentrated seawater as in evaporating tide pools or inland basins like the Dead Sea in Israel or the Salton Sea basin in southern California, ion-poor waters – hypo-ionic to the blood plasma – as most fresh waters, alkaline (soda lakes of East Africa) and naturally acid (e.g. Amazonian) or acidified waters (resulting from anthropogenic or natural sources of pollution), cold, even supercooled (Arctic and Antarctic seas), and warm waters (tropical streams and lakes). Considering that fish inhabit all strata of lakes, seas and oceans it follows that a volume is taken by fish many times larger than that of the terrestrial niche. Remarkably, fishes such as lungfishes, mudskippers, annual fishes and eels (and many others) have developed mechanisms to survive for prolonged times outside the water; lungfishes and mudskippers leave the water to bask in the sun, annual fishes survive periods of drought in mud and clay burrows, the eel encapsulates its gills in a "bag of mucus" and migrates over land when necessary. Imagine the fantastic array of adaptive capacities that fish must have developed already early in evolution to cope with a great variety in environments and that underlie their evolutionary success. In addition, fish were among the first vertebrates to develop complex social structures and behaviours (schooling, symbiosis, hierarchical populations etc.) that must have further contributed to their ecological radiation.

Stress axes in fish

The hypothalamic-pituitary-adrenal (HPA) axis plays a central role in the ability of vertebrates to respond to stressors and give an appropriate adaptive response. Physiology needs to be adjusted to new situations imposed by stressors and to this end an only partly understood neuroendocrine signalling system is activated. In fact, adjustments of physiology are continuously going on, often without being noticed, and whether we score an adjustment as 'adaptation to a stressor' or stress response may only be a matter of definition of gradual differences in the modulation of the HPA/stress axis. The "classical" (i.e. mammalian) hormonal signals that characterise the stress axis are corticotrophin releasing hormone (CRH) produced by neuroendocrine cells in hypothalamic nuclei near the optic chiasm and third ventricle, adrenocorticotropic hormone (ACTH) from the ACTH cells in the pars distalis of the pituitary gland and eventually cortisol or corticosteron from the zona fasciculata of the adrenal cortex. In fish, the end product of this particular axis (called HPI axis as fish have steroid producing interrenal cells in their headkidney rather than adrenal glands) is cortisol. Interestingly, cortisol has both gluco- and mineralocorticoid functions in fish (Wendelaar Bonga 1997, Fig. 1). It is tempting to predict then that all prime targets for the glucocorticoid (liver, muscle, immune system) *and* mineralocorticoid (gills, intestine, kidney) actions of cortisol and the resulting interactions should be considered in the response of the fish to stressors. The first, albeit still circumstantial, evidence that cortisol may work via both glucocorticoid and mineralocorticoid

receptors was very recently published by Sloman & coll (2001), who showed that the proliferation of chloride cells in trout gills is inhibitable by the mineralocorticoid receptor blocker spironolactone.

As in other vertebrates, synthesis and release of cortisol by fish interrenal cells is under primary and acute control of ACTH produced in, and released by distinct cell clusters in the pars distalis of the pituitary gland. In fish though, not every acute stressor evokes an ACTH response preceding cortisol production: seabream exposed to air for 3 min (a typical aquaculture-related stressor) show rises in plasma cortisol but not ACTH (and this contrasts with confinement by increased density that does evoke an ACTH response, Arends *et al.* 1999) and thus other pathways must be active in the stimulation of the interrenal steroid producing cells under particular conditions. Evidence is accruing that in the headkidney of fish the splanchnic nerve runs with pre- and postganglionic fibers through the interrenal compartment; in addition ganglionic cells are found on or in the headkidney that project to the interrenal components (Gallo *et al.* 2001). Thus a neural, sympathetic control of the interregnal cells may explain the cortisol response seen in the absence of an ACTH-surge (Fig. 1).

In addition to the ACTH-cells of the pars distalis, the pars intermedia of the pituitary gland is a source of corticotropic signals, for which α-melanocyte-stimulating hormone (α-MSH) and N-terminally acetylated β-endorphin (N-Ac-β-END) are likely candidates in tilapia, as demonstrated both *in vivo* and *in vitro* (Balm *et al.* 1995, Lamers *et al.* 1992, Lamers *et al.* 1994). The work of Lamers & coll (1994) further suggests a particular role for Thyrotropin Releasing Hormone (TRH) driven α-MSH as a mild corticotrope in adaptation to a long-lasting, unavoidable stressor (low water pH in that study). Clearly, the functions of the hypothalamic and pituitary signals do not obey the role their classical vertebrate name suggests (i.e. being releasing hormone for the pituitary thyrotropes and stimulating hormone for dermal melanophores).

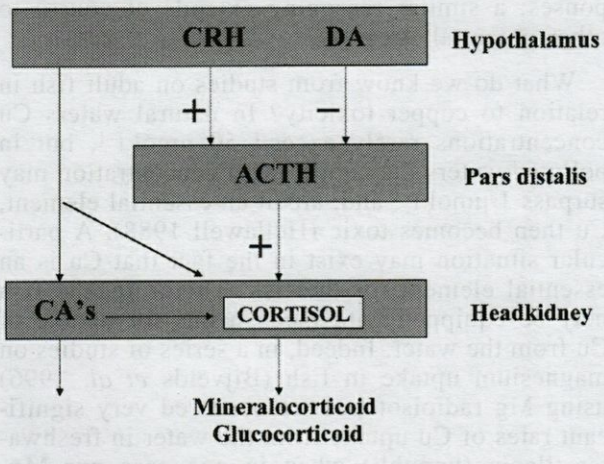

Fig. 1. – Scheme of stress axis activity during acute stress. In the acute stress response catecholamines (CA's) are released via neural pathways. Sympathetic activation leading to CA release may indirectly, via paracrine pathways, and independently from ACTH surges, stimulate cortisol release. The endocrine responses to stressors involve CRH release and inhibition of dopamine (DA) release at the level of the hypothalamus. The stress-induced ACTH-surge from the pituitary corticotropes is a powerful releaser of cortisol from the headkidney steroid producing cells. Cortisol has both glucocorticoid and mineralocorticoid actions, its effects being determined by the steroid receptor make-up of the targets.

An important implication of the particular arrangement of the headkidney – here neural, endocrine, and haematopoietic tissues are found closely together – is that paracrine (Gallo *et al.* 2001) catecholamine release from nerves or neurons following exposure to a stressor may not only activate the chromaffin and steroid producing cells but also the immune system components of this organ (Verburg-van Kemenade *et al.* 2001). And the immune system produces signals such as interleukin-1, tumor necrosis factor ∝, interleukin-6 that affect the neuroendocrine system and thus we can no longer appreciate the stress response of a fish unless we include this notion (Verburg-van Kemenade *et al.* 2001). Evidence was provided that cortisol has immunomodulatory actions on the immune system of carp, e.g. enhancing apoptosis of peripheral lymphocytes but protecting granulocytes from apoptosis (Weyts *et al.* 1998a, Weyts *et al.* 1998b). Such observations substantiate the communication between the neuroendocrine system and the immune system. Very recently, we have found the first in-vitro evidence that carp interleukin 1 stimulates release of MSH and acetylated endorphins from the pars intermedia (Verburg-Van Kemenade *et al.* 2001) and thus the communication between the neuroendocrine and immune system is truly bi-directional (Fig. 2). The mere arrangement of tissues found in fish headkidneys should intrigue and stimulate those involved in comparative studies in the fields of stress physiology and immunology.

Stress in very young fish: a special case for copper as stressor

There is consensus that early life stages of vertebrates are more vulnerable to disturbances than adult stages and this certainly holds true for fishes (von Westerhagen 1988). In fact, nanomolar concentrations of Cu in soft fresh water already disturb the hydromineral balance and the skeletal development of carp larvae (Stouthart *et al.* 1996); adult specimens become intoxicated only when Cu levels

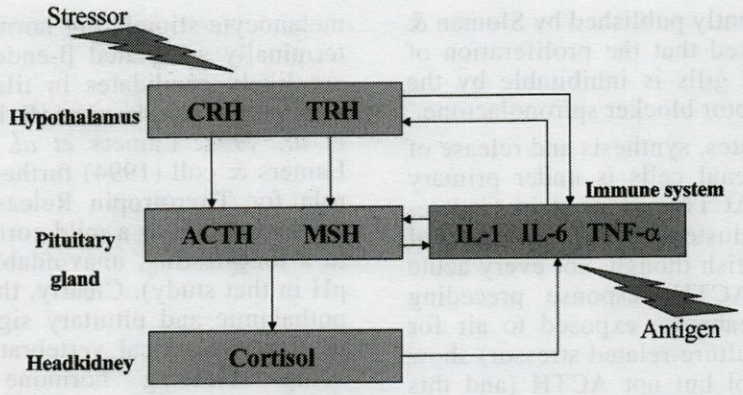

Fig. 2. – Scheme of the stress axis in fish and interaction with immune system.
The CRH, ACTH, cortisol axis (acute responses) and the TRH, MSH, cortisol axis (responses to mild long-lasting and unavoidable stressors) communicate bidirectionally with the immune system, that produces, among others, interleukin-1β (Il-1β) and interleukin-6 (Il-6) and tumor necrosis factor-α (TNF-α) upon stimulation with antigen. The components of the immune system that secure the innate immunity (macrophages, neutrophilic granulocytes, monocytes) are of prime importance for the fish to respond to acute threats. In very young fish this signalling pathway and modulator of the stress axis may be absent for designated time periods.

in the water exceed the micromolar level. With regard to Cu, to date only in *adult* fish an endocrine response to waterborne Cu was demonstrated (Pelgrom *et al.* 1995). The disproportionate sensitivity of fish larvae to toxicants could be attributed to their high surface-to-volume ratio and relatively high metabolic activity. Knowing that hatchlings of fish depend on a large population of integumental (skin, developing gills, yolk sac membrane) chloride cells with a turnover time of around 4 days or less (van der Heijden *et al.* 1999) and considering their crucial role in ion transports and gas exchanges described above for the adult situation, it follows that simply having these cells and their sensitivity to disturbing substances (pollutants, internal metabolites, stress signals) may be the primary cause of the sensitivity of fish early developmental stages to stressors. Alternatively, this sensitivity could relate to an only partly developed HPI-axis in early life stages and incomplete stress response. However, we have recently shown (Stouthart *et al.* 1998) that mere handling of carp eggs elevates the cortisol content. Apparently the embryo already responds with steroid production to handling and it thus follows that the steroid can affect embryonic stages as early as 50 h post-fertilization both in a beneficial and disadvantageous way. It remains to be determined *when* during development the HPI axis of this species becomes fully operational. An important gap in our knowledge exists where it concerns the first occurrence and species of cortisol receptors, as the effects of the steroid eventually depend on the activity of these transcription factors and their specific actions (mineralocorticoid and glucocorticoid receptor actions in fish). Studies on effective feedback by cortisol in early development may be an important tool for the physiologist to get insight in this process.

Knowing now that embryonic stages of fish already give a cortisol response to mechanical (handling) as well as to chemical (Cu) stressors, we have to consider that early exposure to the metal may be a stressful event, even when it is present in concentrations that do not evoke directly visible responses; a similar reasoning extends of course to other potential stressors.

What do we know from studies on adult fish in relation to copper toxicity? In natural waters Cu concentrations rarely exceed 50 $nmol.l^{-1}$, but in polluted waters the copper (Cu) concentration may surpass 1 $\mu mol.l^{-1}$ and, albeit an essential element, Cu then becomes toxic (Hellawell 1988). A particular situation may exist in the fact that Cu is an essential element for the fish. Therefore, the fish may be equipped with mechanisms for uptake of Cu from the water. Indeed, in a series of studies on magnesium uptake in fish (Bijvelds *et al.* 1996) using Mg radioisotopes we observed very significant rates of Cu uptake from the water in freshwater tilapia (unpubli) when in one case our Mg-probe appeared to be contaminated with Cu isotope (this isotope could be discriminated using a Ge/Li detector system). As this uptake was very rapid and constant it occurred in all likelihood via the gills. The chloride cells then would be the prime site to search for the Cu uptake mechanisms as these cells are equipped with the machinery to drive active and regulated ion transports. This being the case, Cu would be a "normal" element for the fish and toxic actions anticipated only at unnaturally high concentrations of Cu; moreover this particular situation would leave a window of Cu concentrations that the researcher could choose to mildly activate

the stress axis. This indeed seems to be possible (see below).

Unfortunately, fresh waters are often polluted, and fish exposed to toxic levels of Cu. As reflected by very significant increases in cortisol release (Pelgrom *et al.* 1995), Cu then apparently acts as a stressor and this is further substantiated by the fact that Cu disturbs the Na^+- and Ca^{2+}-homeostasis of the fish. It does so by a rather specific interaction with the chloride cells (Li *et al.* 1998) and this is, in addition to the reasoning above, another reason to believe that the chloride cells normally pass Cu from water to blood. Waterborne Cu promotes necrosis and apoptosis of branchial chloride cells and respiratory cells (Li *et al.* 1998, Mathiyalagan *et al.* 1996). An important notion in our understanding of Cu toxicity in fish is that fish exposed to waterborn Cu experience an unavoidable and persisting stressor. Fortunately, exogenously administered cortisol enhances the ion transport capacity of fish gills, as it increases the number of chloride cells and elevates the activity of both the Na^+, K^+-ATPase (McCormick 1995) and Ca^{2+}-ATPase (Flik & Perry 1989, Flik *et al.* 1995) activities in these cells. One may anticipate, therefore, that cortisol will ameliorate or even counteract effects of Cu on these cells, at least partly. Furthermore, cortisol protects chloride cells against necrosis as a gill filament culture pre-incubated with 0.3 $\mu mol.l^{-1}$ cortisol exhibits a 75% reduction in chloride cell necrosis when the tissue is exposed to 100 $\mu mol.l^{-1}$ Cu (Bury *et al.* 1997). Thus, prevention of ionoregulatory disturbances through actions on chloride cells appears to be a major function of the fish's endocrine stress response evoked by Cu, or any stressor that interferes with ion regulation and impinges on chloride cell functioning. Cu is in this respect an interesting stressor to study as its targets and effects are becoming increasingly clear.

When exposed to Cu, ACTH and cortisol contents in very early stages (48 hours post fertilization) of carp rise; this demonstrates that at least the peripheral components of the hypothalamo-pituitary-interrenal (HPI-) axis are active in embryonic stages (Flik *et al* 2001). This observation is in perfect agreement with an earlier study (Stouthart *et al.* 1998), where it was shown that pre-hatch stages of carp (50 hours post fertilization) produce cortisol in response to handling. We thus may conclude that the pituitary-interrenal axis becomes responsive and probably functional in this species before hatching. The very early response may be typical of carp as in some other species of fish cortisol production could be demonstrated only in later stages of development, i.e. as off 24 hours post hatching (DeJesus *et al.* 1991, DeJesus & Hirano 1992, Hwang *et al.* 1992, Hwang & Wu 1993, Barry *et al.* 1995, Sampath-Kumar *et al.* 1995). For handling of eggs and early hatchlings in aquaculture settings it may be beneficial to know

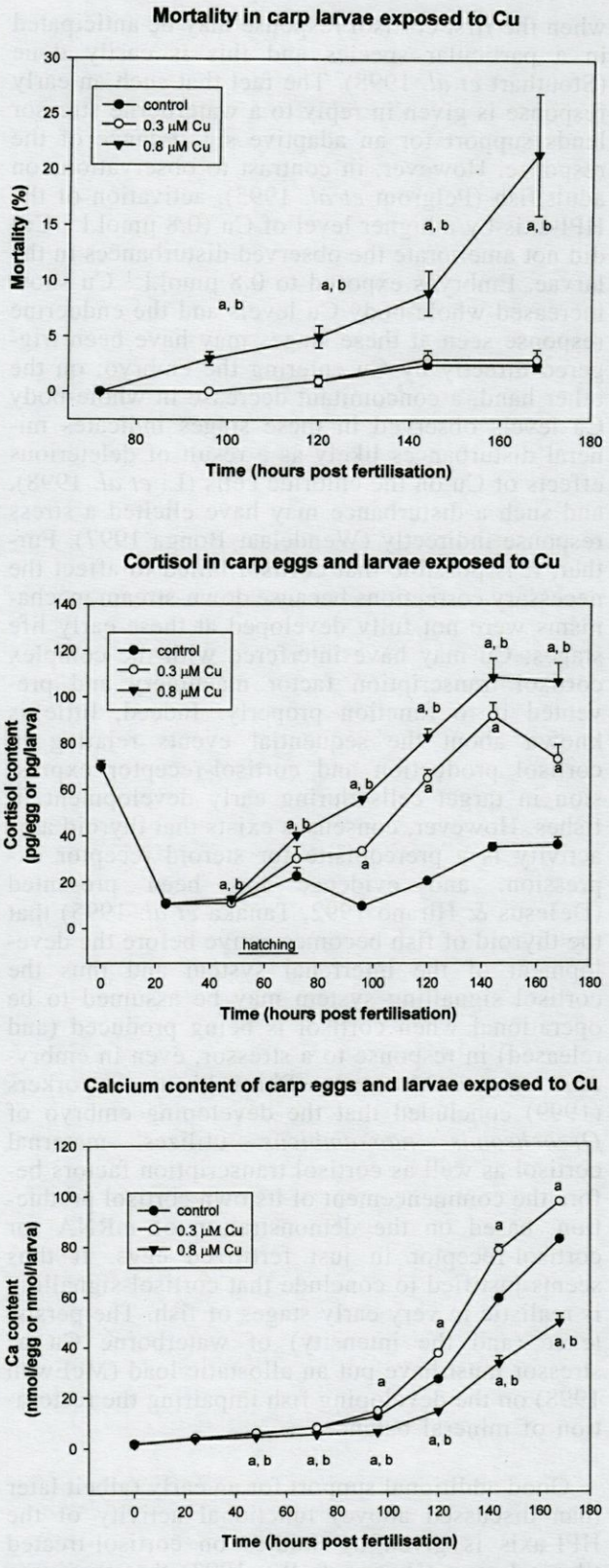

Fig. 3. – Mortality (a), cortisol (b) and Ca content (c) in carp larvae exposed to two levels of Cu (0.3 and 0.8 $mol.l^{-1}$). Data are expressed as means of 6 experiments and bars indicate SEM. Letters signify differences among groups at the 5% level, a compared to controls, b compared to the group exposed to the low concentration of Cu (data taken in modified form from Flik *et al.* 2001).

when the first cortisol response may be anticipated in a particular species and this is easily done (Stouthart *et al.* 1998). The fact that such an early response is given in reply to a waterborne stressor lends support for an adaptive significance of the response. However, in contrast to observations on adult fish (Pelgrom *et al.* 1995), activation of the HPI-axis by a higher level of Cu (0.8 $\mu mol.l^{-1}$ Cu) did not ameliorate the observed disturbances in the larvae. Embryos exposed to 0.8 $\mu mol.l^{-1}$ Cu show increased whole-body Cu levels and the endocrine response seen at these stages may have been triggered directly by Cu entering the embryo; on the other hand, a concomitant decrease in whole-body Ca levels observed in these stages indicates mineral disturbances likely as a result of deleterious effects of Cu on the chloride cells (Li *et al.* 1998), and such a disturbance may have elicited a stress response indirectly (Wendelaar Bonga 1997). Further, it is possible that cortisol failed to affect the necessary corrections because down-stream mechanisms were not fully developed at these early life stages; Cu may have interfered with the complex cortisol transcription factor machinery and prevented it to function properly. Indeed, little is known about the sequential events relating to cortisol production and cortisol-receptor expression in target cells during early development of fishes. However, consensus exists that thyroid axis activity is a prerequisite for steroid receptor expression, and evidence has been presented (DeJesus & Hirano 1992, Tanaka *et al.* 1995) that the thyroid of fish becomes active before the development of the interrenal system and thus the cortisol signalling system may be assumed to be operational when cortisol is being produced (and released) in response to a stressor, even in embryonic stages. Moreover, Shiraishi an coworkers (1999) concluded that the developing embryo of *Oreochromis mossambicus* utilizes maternal cortisol as well as cortisol transcription factors before the commencement of its own cortisol production, based on the demonstration of mRNA for cortisol-receptor in just fertilized eggs. It thus seems justified to conclude that cortisol-signalling is realistic in very early stages of fish. The persistence (and the intensity) of waterborne Cu as stressor must have put an allostatic load (McEwen 1998) on the developing fish impairing the restoration of mineral balance.

Good additional support for an early (albeit later than discussed above) functional activity of the HPI-axis is given by studies on cortisol-treated tilapia larvae (Hwang & Wu 1993) that survive a transfer from fresh water to 27.5 ‰ seawater better than did larvae which did not receive exogenous cortisol. Exogenous cortisol given to 1-day old tilapia enhances growth and development (Mathiyalagan *et al.* 1996). But one cannot exclude that exogenous cortisol impairs, by feedback, the ACTH release normally seen in response to a stressor and thus that an early cortisol response was artificially suppressed. The fact that growth and development were not disturbed in the experiments of Mathiyalagan and colleagues lends support for a physiological effect of cortisol in their experiments. Carefully chosen concentrations of Cu (0.3 $\mu mol.l^{-1}$; 0.8 $\mu mol.l^{-1}$ being already too high) elicit an cortisol response in very young carp without disturbance of mineral balance an example that cortisol/stress responses can be ameliorative and need not be deleterious (Flik *et al.* 2001). Larvae exposed to 0.3 $\mu mol.l^{-1}$ Cu had an elevated whole-body Ca content suggesting a stimulatory effect of cortisol on chloride cell activity as these cells are primarily involved in calcium uptake from the water. The observation that 0.3 $\mu mol.l^{-1}$ Cu does not affect the Na content of carp larvae suggests that sodium and calcium uptake are at least partly dissociated or that sodium uptake mechanisms associated with the chloride cells are more vulnerable to Cu exposure (Na^+ transport mechanisms being stimulated by cortisol and inhibited by Cu, resulting in net unaltered activity). This would be in line with conclusions based on in-vitro (Li *et al.* 1996) as well as in-vivo data (Li *et al.* 1998). It follows then that the *higher* Cu concentration (0.8 $\mu mol.l^{-1}$) poisoned chloride cells, inhibited both calcium and sodium uptake mechanisms and prevented a potential ameliorative action of cortisol leading to a decrease in whole body calcium content. Taken together these results indicate that cortisol is involved in the defence against Cu toxicity inflicted upon the chloride cells of the integument. Cortisol was shown to induce synthesis of the heavy metal-sequestering protein metallothionein in chloride cells specifically; this forms part of the adaptive response to toxic effects of high levels of Cu and again points to the chloride cells as important targets in the actions of cortisol (Dang *et al.* 1999).

The question arises how the (H)PI-axis becomes activated, in particular at lower Cu concentrations. Rainbow trout avoid water containing 2 $nmol.l^{-1}$ Cu (Folmar 1976), a concentration which is below that reported in some natural waters (Hellawell 1988) and not considered toxic at all. After hatching larvae are in direct contact with the ambient water and olfactory perception may allow the newly-hatched larva to trigger the HPI-axis, provided the connections between hypothalamus and pituitary have become functional to pass on the olfactory messages. One cannot exclude however, that as yet unknown signals produced by chloride cells or other mediator cells (macrophages etc.) become systemic and activate the stress axis. Cu *per se* can activate the genes coding for metallothionein and it could, therefore, trigger extracellular signals from these cells as well, an option open for future research.

Although exposure to low levels of Cu (0.3 $\mu mol.l^{-1}$) does not seem to disturb embryonic or larval physiology acutely, one cannot exclude the possibility that artificially increased cortisol levels near hatching can not have adverse effects on the subsequent development of the fish. In mammals prenatal stress alters offspring behaviour, morphology and physiology (Weinstock 1997) and modifies the stress response of the young (Meaney *et al.* 1996). Prenatally stressed human infants and rats show attention deficits, hyperanxiety and disturbed social behaviour during later life (Williams *et al.* 1998). Considering the sensitivity of early life stages, it is likely that stress experienced during embryonic and larval stages in fish has implications for stress-responsiveness during later life. This may be a concern for those involved in fish aquaculture and ecophysiological research.

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