Stress responsiveness of the pituitary–interrenal axis during early life stages of common carp (*Cyprinus carpio*)

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Abstract

Whole-body levels of ACTH, α -MSH and cortisol in eggs and larvae of the common carp (*Cyprinus carpio*) were determined periodically up until 168 h after fertilisation. ACTH, α -MSH and cortisol immunoreactivity was detected in unfertilised eggs, and endogenous production of ACTH and α -MSH was observed 24 h after fertilisation and that of cortisol 36 h after fertilisation. ACTH immunoreactivity reached peak levels before hatching (56–72 h after fertilisation) and remained relatively stable thereafter, while α -MSH immunoreactivity started to increase after hatching. At 36 h after fertilisation, whole-body cortisol levels increased rapidly reaching peak levels at the end of hatching (72 h after fertilisation), remaining stable until the end of the experiment. From 50 h after fertilisation onwards, embryos and larvae increased their whole-body cortisol levels when subjected to handling (mechanical pressure during egg stage or netting during the larval stage). It is concluded that the pituitary–interrenal axis in carp is fully functional at the time of hatching. No indications of a stress non-responsive period after hatching were observed.

To characterise ACTH and α -MSH immunoreactivities in carp larvae, whole-body homogenates were analysed by HPLC, with pituitary homogenates of adult carp serving as a reference. ACTH and α -MSH immunoreactivity in carp larvae homogenates consisted of three and two products respectively. HPLC of adult carp pituitaries revealed the presence of two ACTH immunoreactive products, which may represent a phosphorylated and a non-phosphorylated ACTH variant, while the three α -MSH peaks most likely represent des-acetylated, mono-acetylated and di-acetylated α -MSH, the latter being the predominant form. In carp larvae, however, one of the ACTH immunoreactive products co-eluted with the non-phosphorylated ACTH, while the two α -MSH products identified co-eluted with des-acetylated and mono-acetylated α -MSH, indicating that POMC processing at this stage of development is different from prohormone processing in adult fish.

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Introduction

The hypothalamus–pituitary–interrenal (HPI) axis of fish is crucial for the adaptation of these animals to environmental challenges. Adrenocorticotrophic hormone (ACTH), which is produced in the pars distalis of the pituitary gland, is considered the major corticotrophic factor regulating the synthesis and release of cortisol (Balm *et al.* 1994, Wendelaar Bonga 1997). In tilapia (*Oreochromis mossambicus*), α -melanocyte-stimulating hormone (α -MSH), which is produced in the pars intermedia of the pituitary gland, exerts corticotrophic actions *in vitro* (Lamers *et al.* 1992). The interrenal corticosteroid cortisol has been shown to affect osmoregulation, energy metabolism, immune competence (Wendelaar Bonga 1997) and developmental processes, including hatching, growth and metamorphosis, in particular during stress (De Jesus *et al.*

1991, Yamano *et al.* 1991). The presence of cortisol has been demonstrated in eggs and larvae of various cichlid and salmonid species (De Jesus & Hirano 1992, Hwang *et al.* 1992, Hwang & Wu 1993, Barry *et al.* 1995). In unfertilised fish eggs, cortisol is already present because of maternal transfer during oogenesis (Feist *et al.* 1990), but endogenous cortisol production does not start before hatching (De Jesus *et al.* 1991, Hwang *et al.* 1992, Hwang & Wu 1993, Barry *et al.* 1995, Sampath-Kumar *et al.* 1995). Studies on salmonid species have indicated that ACTH as well as α -MSH immunopositive cells are present in the pituitary glands of fish embryos (Naito *et al.* 1993, Saga *et al.* 1993), although the onset of production of these hormones and their concentrations have not yet been determined in these life stages.

In early stages of development, the HPI axis is activated during stress. Studies on salmonids using temperature

shock and handling showed that the axis could be activated after hatching (Pottinger & Mosuwe 1994, Barry *et al.* 1995). In other studies, toxic stressors were used to induce a stress response. Stouthart *et al.* (1998) showed that carp larvae exposed to PCB 126 evoked a stress response 3 days after hatching. Stephens *et al.* (1997) showed that turbot (*Scophthalmus maximus*) larvae exposed to the water-soluble fraction of crude oil increased their whole-body cortisol levels 2 days after hatching. In most of these studies, cortisol was the factor studied, whereas the role of ACTH and α -MSH has received much less attention.

The aim of this paper is twofold. First to follow ACTH, α -MSH and cortisol production during embryonic and larval development of carp (*Cyprinus carpio*) until 168 h after fertilisation, and, secondly, to determine at what time embryos and larvae can respond to handling stress by elevating whole-body cortisol levels.

Materials and Methods

In vitro fertilisation and incubation of eggs

Carp gametes were obtained from the common carp (*Cyprinus carpio*) as described by Oyen *et al.* (1991). Approximately 1200 eggs from one female were fertilised by sperm from one male and distributed among 20 Petri dishes. At 1 min after fertilisation, the Petri dishes, with fertilised eggs stuck to the bottom, were rinsed and transferred to 4-litre aquaria through which oxygenated UV-sterilised chemically defined water was pumped at 1 litre/h at 24 °C under a 12 h light/12 h darkness cycle. The water contained the following ions (mmol/l): 0.06 KCl; 0.40 NaHCO₃; 0.20 MgSO₄; 0.80 CaCl₂. Eggs were considered dead when parts of the content turned opaque and white, or when the heart beat had stopped. Dead eggs were immediately removed to prevent fungal infection of the other eggs. The larvae were not fed during the experiment. Directly after fertilisation, 10 Petri dishes, each containing at least 30 eggs, were put in separate aquaria, and embryos or larvae were sampled at 12, 24, 36, 48, 60, 72, 96, 120, 144 and 168 h after fertilisation for determination of wet weight and hormone concentrations ($n=20$). Two Petri dishes containing a total of at least 350 eggs were put in one aquarium for HPLC analysis, histology and immunology at 168 h after fertilisation. For the handling experiments, five Petri dishes, each containing at least 10 eggs, were put in one aquarium and used 50 h after fertilisation. Three Petri dishes (at least 150 eggs per dish) were kept in separate aquaria to carry out handling experiments with larvae at 72, 96 and 120 h after fertilisation. This protocol was repeated five times, yielding six determinations per treatment.

Histology

At 168 h after fertilisation, larvae were collected for light microscopical examination. After fixation in Bouin's

solution, dehydration, and embedding in paraffin, sagittal sections were cut at 5 μ m thickness. An immunocytochemical method using antisera raised in rabbits against human ACTH(1–24) (this antiserum also detects melanotropes; van Eys & van den Oetelaar 1981) and α -MSH (van Zoest *et al.* 1989) was used to identify immunoreactive ACTH and α -MSH cells.

RIAs

Concentrations of ACTH, α -MSH and cortisol were determined in duplicate by RIA as described by Balm *et al.* (1994), van Zoest *et al.* (1989) and Balm *et al.* (1998) respectively. The ACTH antibody had negligible cross-reactivity with α -MSH. The α -MSH antibody recognised des-acetylated, mono-acetylated and di-acetylated α -MSH with similar sensitivity. Although we cannot exclude cross-reactivity with components in the homogenates, the cortisol antibody has marginal cross-reactivity with 11-deoxycortisol (5.9%), cortisone acetate (0.16%), cortisone (2.6%) and 17 α -hydroxyprogesterone (0.4%). The detection limit of the assay was per tube: 0.32 pg ACTH, 2.5 pg α -MSH and 4 pg cortisol. The intra- and inter-assay coefficients of variation were 6 and 7% for ACTH, 5 and 11% for α -MSH, and 3 and 5% for cortisol respectively.

Biochemistry

To identify different ACTH and α -MSH immunoreactive forms, 300 larvae (168 h after fertilisation) were homogenised in 1 ml ice-cold 0.01 M HCl using a glass-to-glass Potter homogeniser. Membranes and particulate material were removed by centrifugation (13 000 g, 10 min; repeated three times). The peptides were separated by reverse-phase HPLC. Fractions were freeze-dried under vacuum (Speed-Vac Concentrator Savant; New Brunswick Scientific, Edison, UK) and redissolved in 150 μ l RIA buffer. ACTH and α -MSH immunoreactivity were determined in duplicate by RIA.

Development

To determine wet weight, 20 eggs or larvae from each batch were collected at 0 h (unfertilised eggs), 12, 24, 36, 48, 60 and 72 h (hatching at 56–72 h after fertilisation), 96, 120 and 144 h (yolk absorption completed) and 168 h after fertilisation. Eggs or larvae were frozen in a mixture of dry ice and methanol (approximately –70 °C), collected in preweighed vials and weighed. To these samples were added 200 μ l (0, 12, 24, 36, 48, 72 and 96 h after fertilisation) or 400 μ l (120, 144 and 168 h after fertilisation) 0.01 M ice-cold HCl, and the mixture was subsequently homogenised using a glass-to-glass Potter homogeniser. Membranes and particulate

Figure 1 Mid-sagittal sections through the pituitary gland of 168 h old carp larvae (magnification $40\times$). (a) Immunoreactive ACTH in cells located in the pars distalis (pd) and immunoreactive melanotrope in the pars intermedia (pi) of the pituitary. (b) Immunoreactive α -MSH in cells located in the pars intermedia (pi) of the pituitary.

material were removed by centrifugation ($13\,000\text{ g}$, 10 min; repeated three times). Whole-body ACTH, α -MSH and cortisol levels were determined in duplicate by RIA.

Handling

At 50 h after fertilisation a total of five Petri dishes were used. Embryos of one Petri dish were sampled at rest (time 0) by transferring them to a mixture of dry ice and methanol. Embryos of the remaining Petri dishes were mechanically disturbed by squeezing the eggshell slightly with forceps, inducing one tail movement of the embryo. This procedure started at rest (time 0) and was repeated after 1, 2, 3 and 4 min. Embryos were sampled after 5, 30, 60 and 120 min. A similar experiment was carried out with larvae at 72, 96, 120 h after fertilisation. At each of these time points 20 larvae were sampled at rest (time 0). The remaining larvae were netted for 1 min and distributed among four Petri dishes and subsequently sampled 5, 30, 60 and 120 min afterwards. To the samples were added 200 μl (50, 72 and 96 h after fertilisation) or 400 μl (120 after fertilisation) 0.01 M ice-cold HCl, and the samples were subsequently homogenised. Whole-body levels of cortisol were determined in duplicate by RIA. This experiment was repeated five times, yielding six determinations.

Statistical analysis

Differences among variances were evaluated by the Bartlett test. Mann–Whitney tests were used to assess differences among means. In data presented in Fig. 4, means of the time point following directly after the lowest hormone value were tested. Statistical signifi-

cance was accepted at $P<0.05$. Data are expressed as means $\pm$ S.E.M.

Results

Histology

Immunoreactive ACTH and α -MSH cells in the pituitary of 168 h old carp larvae are shown in Fig. 1. At 168 h after fertilisation, immunoreactive ACTH could be identified in cells located in the distal lobe of the pituitary (Fig. 1a). ACTH immunopositivity was also observed in the melanotrope cells. Immunoreactive α -MSH was specifically located in cells of the intermediate lobe of the pituitary (Fig. 1b).

Biochemistry

Dose–response competitive binding curves for standard ACTH, α -MSH and cortisol extracts of carp eggs (48 h after fertilisation) and larvae (168 after fertilisation) are shown in Fig. 2. Serial dilutions of egg and larvae homogenates displaced radiolabelled ACTH, α -MSH and cortisol from the antibody in parallel with dilutions of the standards used.

HPLC analyses of the ACTH and α -MSH immunoreactivities in carp pituitary homogenates are shown in Fig. 3. ACTH was present as two products (peak A and B), peak B being the predominant form. The α -MSH immunograms from the adult carp pituitary homogenates demonstrated three peaks, designated des-acetylated, mono-acetylated and di-acetylated α -MSH (Follénus *et al.* 1985). In carp larvae homogenates, three ACTH products were seen. Only one peak (termed A') was co-eluted with peak A in the adult carp pituitary. Analysis

Figure 2 Competitive binding curves of ACTH, α -MSH and cortisol standards and dilution curves of ACTH, α -MSH and cortisol in homogenates of eggs (48 h after fertilisation) and larvae (168 h after fertilisation). B/B_o , bound/free.

of the α -MSH immunoreactivity in carp larvae homogenates revealed two products, co-eluted with des-acetylated and mono-acetylated α -MSH.

Development

The wet weight of 20 unfertilised eggs was 23.9 ± 0.6 mg. Directly after fertilisation, it increased to 56.4 ± 2.9 mg

and remained stable until hatching. Thereafter, the wet weight of 20 larvae decreased to 27.5 ± 1.6 mg and remained stable until 168 h after fertilisation. Whole-body immunoreactive ACTH, α -MSH and cortisol levels during the first 168 h of carp development are shown in Fig. 4. ACTH immunoreactivity could be detected in unfertilised eggs ($t=0$) and decreased until 24 h after fertilisation. From 24 h after fertilisation, ACTH

Figure 3 HPLC immunograms of adult carp pituitaries and whole-body carp larvae homogenates (168 h after fertilisation). ●, Elution profiles of ACTH and α -MSH. Open and closed arrows indicate the elution position of the human ACTH(1–39) standard and α -MSH standard respectively. Between 10 and 22 min, 0.3 min fractions were collected.

immunoreactivity increased significantly ($P < 0.05$). In particular, before and during hatching (56–72 h after fertilisation), ACTH immunoreactivity increased markedly. After hatching, ACTH levels decreased. Immunoreactivity of α -MSH could be detected in unfertilised eggs ($t=0$); it decreased until 24 h after fertilisation, and then increased slightly but significantly ($P < 0.001$). After hatching, whole-body α -MSH levels rapidly increased further and stabilised. In unfertilised eggs, high levels of cortisol were present, which decreased over the 36 h post-fertilisation period. After that, whole-body cortisol levels significantly

($P < 0.001$) increased to a steady level at 72 h (end of hatching).

Handling

Whole-body cortisol levels of controls and stressed carp embryos and larvae at 50, 72, 96 and 120 h after fertilisation are shown in Fig. 5. Embryos (50 h after fertilisation; 6 h before hatching) responded 5 min after handling with a significant ($P < 0.01$) increase in whole-body cortisol levels, which returned to control levels within 30 min.

Figure 4 Changes in ACTH, α -MSH and cortisol immunoreactivity (in pg per 20 eggs or larvae) during the early development of carp. Data are expressed as means $\pm$ S.E.M. ($n=6$).

Directly after hatching (72 h fertilisation), larvae subjected to handling had significantly ($P<0.01$) increased cortisol levels at 5, 30 and 60 min, decreasing to control levels after 120 min. Larvae subjected to handling at 96 or 120 h after fertilisation had significantly increased whole-body cortisol levels compared with controls at respectively 5 ($P<0.01$),

or 5 and 30 min ($P<0.05$), and returned to control levels after 30 or 60 min respectively.

Discussion

Characterisation of ACTH and α -MSH

As in tilapia (Balm *et al.* 1994), two immunoreactive forms of ACTH (termed A and B) were found in the pituitary of adult carp. The antiserum used has been demonstrated to detect ACTH molecules in a variety of (non-mammalian) vertebrate species (Dores *et al.* 1993). The immunoreactive ACTH products in carp were eluted earlier than the human ACTH standard, which has also been demonstrated for tilapia (Balm *et al.* 1994). In the latter study it was demonstrated that the two ACTH immunoreactive products were equipotent in stimulating cortisol release from the interrenal cells, their potency being similar to that of human ACTH. We therefore conclude that the two products identified in the present study represent carp ACTH. Consistent with this, Hon & Ng (1986) showed that several fractions of carp pituitary glands separated via CM-cellulose chromatography possessed ACTH-like immunoreactivity, whereas only two fractions were bioactive, as evidenced by dose-related stimulation of corticosterone production from isolated rat adrenal cells. Tran *et al.* (1989) found two immunoreactive ACTH products in the cyprinid goldfish (*Carassius auratus*). Also in human pituitaries, two distinct, steroidogenically equipotent forms of ACTH were isolated and purified (Bennett *et al.* 1983). This latter study identified the more polar form of ACTH as a phosphorylated form, whereas quantitatively the non-phosphorylated ACTH was most abundant. Possibly, the two products found in adult carp pituitaries also represent a non-phosphorylated (A) and a phosphorylated (B) form of ACTH, as discussed previously for tilapia (Balm *et al.* 1994). The finding that teleost ACTH, but not α -MSH, is eluted at different positions from their human counterparts most likely relates to the considerable dissimilarities in amino acid sequence between the hormones, which is almost exclusively restricted to the corticotrophin-like intermediate lobe peptide (CLIP) moiety. Three forms of α -MSH were found in the adult carp pituitary, i.e. des-acetylated, mono-acetylated and di-acetylated. These α -MSH forms were described previously for the intermediate lobes of the cyprinids *Cyprinus carpio* and *Carassius auratus* by Follénus *et al.* (1985), and the cichlid tilapia by Lamers *et al.* (1991) and Balm *et al.* (1994). In agreement with their findings, the di-acetylated α -MSH was most abundant in the melanotropes of our carp. Because no literature is available on immunoreactive forms of ACTH and α -MSH during the early life stages of the common carp and other teleosts, we compared data with the HPLC profiles of pituitaries obtained from adult carp pituitaries. The analysis of 168 h

Figure 5 Changes in cortisol immunoreactivity at 5, 30, 60 and 120 min after handling stress during different stages of the early development of carp (pg per 20 eggs or larvae): 50 h after fertilisation (before hatching); 72 h after fertilisation (directly after hatching); 96 and 120 h after fertilisation. Data are expressed as means $\pm$ S.E.M. ($n=6$).

old carp larvae revealed three ACTH immunoreactive peaks. One product (A'), co-eluted with peak A in the adult carp pituitary, may represent the non-phosphorylated form of ACTH. Although the two other peaks have not been identified, they could either originate from a larval type of corticotrope as present in mammals (Perez *et al.* 1997) or they could reflect different pro-opiomelanocortin processing in embryonal and mature corticotropes. The ACTH antibody used in our experiments showed a marginal cross-reactivity with CLIP in other vertebrates (R. M. Does, personal communication) but negligible cross-reactivity with tilapia CLIP (Balm *et al.* 1994). The unidentified peaks therefore probably do not represent carp CLIP. Two α -MSH immunoreactive

products were found in these carp larvae, co-eluted with des-acetylated and mono-acetylated α -MSH respectively. Although no di-acetylated form was found at this stage of development, these findings show that acetylation of α -MSH is taking place at least 168 h after fertilisation.

Structural development of the HPI axis

The results of this study demonstrate that in carp the pituitary–interrenal axis is already functional at the time of hatching (56 h after fertilisation). This conclusion is based on three observations. First, whole-body ACTH (24 h after fertilisation), cortisol (36 h after fertilisation), and to a

lesser extent, α -MSH levels markedly increased before hatching. Secondly, elevated whole-body cortisol levels were observed in carp embryos before hatching as a result of handling. Thirdly, carp larvae responded to handling stress directly after hatching, indicating that a non-stress-responsive period after hatching is absent. This is the first study to measure concurrently whole-body levels of ACTH, α -MSH and cortisol during the early development of a freshwater fish and after exposure to handling stress in this period. Previously we showed (Stouthart *et al.* 1998) that a 48 h exposure to the toxicant PCB 126 during the embryonic stage induced an increase in whole-body ACTH, α -MSH and cortisol levels in carp larvae compared with larvae from sham-treated embryos.

The presence of ACTH, α -MSH and cortisol in unfertilised eggs and the first embryonic stages of teleosts is most likely due to maternal deposition of these hormones in the yolk sac (Feist *et al.* 1990). In several fish species, plasma cortisol levels are rising during the final stages of vitellogenesis and during ovulation, and cortisol is known to pass easily through cell membranes because of its lipophilicity (Feist *et al.* 1990). Also the presence of other lipophilic hormones such as thyroid hormones and sex steroids like oestradiol and testosterone have been demonstrated in eggs of teleosts (Feist *et al.* 1990, De Jesus *et al.* 1991, De Jesus & Hirano 1992). However, peptide hormones such as prolactin and growth hormone have also been demonstrated in fish eggs (Lam 1994). A possible reason is that ACTH and α -MSH are transferred passively with vitellogenin into the eggs during oogenesis, but the typical characteristics of the larval ACTH and α -MSH immunoreactivities may argue against this. The function of the hormones deposited by the female in the eggs is not yet clear, although the patterns of disappearance of the various hormones from the yolk may reflect the demand of the embryos for these hormones during embryogenesis, until endogenous hormone production starts (Brown & Bern 1989). Endogenous ACTH and α -MSH production, as judged from an increase in whole-body hormone content, was detected biochemically 24 h after fertilisation. Since no literature on whole-body levels of ACTH and α -MSH in early stages of fish development is available, our results can only be compared with immunohistochemical data. Naito *et al.* (1993) detected immunoreactive ACTH and α -MSH in the pituitary of chum salmon (*Oncorhynchus keta*) 2 weeks before hatching. The number, size and also the intensity of the immunoreactive staining of the ACTH and α -MSH cells increased gradually during embryonic life, and markedly after hatching (Naito *et al.* 1993, Saga *et al.* 1993). These authors found a coordinated development of the adeno- and neurohypophysis. Neuroendocrine fibres, from areas including the preoptic regions, reached the neurohypophysis during the final week of embryonic life, indicating the development of the characteristic hypothalamus-pituitary

relationship by the time of hatching. In a study on the early development of rainbow trout (*Oncorhynchus mykiss*), Saga *et al.* (1993) found immunoreactive α -MSH at 18 days after fertilisation (stage 21) and ACTH at 35 days after fertilisation (stage 28) in the pituitary gland; hatching occurred 42 days after fertilisation (stage 29). Both number and immuno-reactivity of the cells increased gradually during embryonic development. In most of these studies the distinction between the production sites of both hormones, in particular in embryos, could not be clearly established, because the ACTH and α -MSH antibodies used showed some cross-reactivity, and because before hatching the ACTH- and α -MSH-producing cells are still located in the same area of the pituitary gland. The decrease in whole-body ACTH and α -MSH during the early embryonic stages indicates the clearance of these hormones. No literature is available on the clearance of ACTH and α -MSH during early development. Several studies on different fish species showed that, in fertilised eggs, corticosteroids as well as sex steroids and thyroid hormones can be metabolised (De Jesus *et al.* 1991, De Jesus & Hirano 1992, Hwang *et al.* 1992, Hwang & Wu 1993, Barry *et al.* 1995, Sampath-Kumar *et al.* 1995). Importantly, endogenous ACTH and α -MSH production occurred in the carp embryos before cortisol production had started. After hatching, whole-body ACTH levels decreased slightly but remained relatively stable, while whole-body levels of α -MSH increased markedly 96 h after fertilisation.

The rapid decrease in cortisol that we observed during the first stages of embryonic development in carp has also been reported for Japanese flounder, chum salmon, tilapia, rainbow trout and Asian seabass (*Lates calcarifer*) (De Jesus *et al.* 1991, De Jesus & Hirano 1992, Hwang *et al.* 1992, Hwang & Wu 1993, Barry *et al.* 1995, Sampath-Kumar *et al.* 1995). This points to metabolisation in the embryos of the cortisol of maternal origin. As mentioned above, teleost embryos have been shown to metabolise steroids from the day of fertilisation of the eggs, and we suggest that eggs may utilise maternal cortisol during early development as long as the interrenal gland is not yet functional. Most authors showed that whole-body cortisol levels decreased during the whole course of embryonic development of different species (De Jesus *et al.* 1991, Hwang *et al.* 1992, Hwang & Wu 1993, Barry *et al.* 1995, Sampath-Kumar *et al.* 1995), and these authors could not demonstrate endogenous cortisol production before hatching. In contrast, our observations on carp show that the levels of this hormone markedly increased 36 h after fertilisation, which demonstrates that endogenous production had already started well before hatching. Thus carp is the first species for which endogenous production of ACTH, α -MSH as well as cortisol has been demonstrated at the embryonic stage. The first indications of endogenous cortisol production in other fish

species have only been observed after hatching: tilapia after 1 day (Hwang *et al.* 1992, Hwang & Wu 1993); rainbow trout after 1 day (Hwang *et al.* 1992, Barry *et al.* 1995); milkfish (*Chanos chanos*) after 3 days (Hwang *et al.* 1992); Asian seabass after 4 days (Sampath-Kumar *et al.* 1995); yellowfin bream (*Acanthopagrus latus*) after 5 days; and Japanese flounder after 15 days (De Jesus *et al.* 1991).

Functional development of the HPI axis

Typically, the first signs of cortical tissue in teleosts are observed before hatching (Chester Jones & Mosley 1980), which is consistent with our finding that whole-body cortisol levels increased within 5 min when carp embryos were stressed. This indicates that a stress response can already be evoked at a very early stage in the life of the carp. However, no evidence was found that the HPI axis of rainbow trout is transiently activated before hatching on handling stress (Pottinger & Mosuwe 1994, Barry *et al.* 1995). Our experiments also show that freshly hatched larvae were sensitive to handling. In salmonids this response appears after hatching. In rainbow and brown trout (*Salmo trutta*) a rise in whole-body corticosteroids in response to handling and confinement could not be observed before larvae reared at 5 °C were 5 weeks old, and it took 30–60 min to develop (Pottinger & Mosuwe 1994). Barry *et al.* (1995) demonstrated doubling of cortisol levels in 2-week-old post-hatch rainbow trout larvae reared at 10 °C, 1 h after 30 s handling followed by 0 °C cold shock. These results indicate that the start of the cortisol stress response might be related to the temperature at which the fish eggs and larvae have been reared. Stephens *et al.* (1997) showed that, in turbot (*Scophthalmus maximus*) larvae that were kept at 15 °C and exposed to crude oil, whole-body cortisol levels increased from as early as 2 days after hatching. A response by the HPI axis to a stressor in fish embryos, as demonstrated in our study on carp, is so far unique to fish. In birds, stress responsiveness at first gradually increases but markedly rises just before hatching (Carsia & Malamed 1989). This is followed by a period of non-responsiveness to stressors, which starts after hatching and lasts for 48 h (DeKloet *et al.* 1988). Similarly, in mammals a period of non-responsiveness has been reported immediately after birth. In rats, basal corticosteroid levels decrease dramatically after birth and remain low for 2 weeks, during which time different types of stressor do not elicit increases in circulating glucocorticoid levels as they do in adult animals (DeKloet *et al.* 1988). We obtained no evidence for a period of non-responsiveness to stressors during the early development of carp. The species differences in HPI development between carp and other species may correlate with the differences in the morphogenetic stages of teleost larvae at the time of hatching (Balon 1981) and may not be solely temperature-

dependent. Also, differences between the early life histories of salmonids and carp could relate to the differences observed, since salmonids spend a considerable time period as yolk-sac larvae or alevins within the gravel substrate in which the eggs are laid, whereas cyprinids are free-swimming larvae on hatching. Carp is of interest because its embryonic development is very fast (72 h at 24 °C), much faster than, for instance, the development of a warm water species such as tilapia (12 days at 26 °C) or cold water species such as salmonids (27–29 days at 10 °C).

The cortisol response to handling in carp embryos was observed at a time when ACTH and α -MSH production has already started, and, since both hormones have been implicated in the pituitary control of cortisol secretion in fish, one or both may have acted as the cortisol secretagogue in this experiment. In addition to short-term regulation of cortisol, the trophic action of ACTH is also probably involved in the normal development of the interrenal cells (DeKloet *et al.* 1988). α -MSH appears to be a less likely cortisol secretagogue at this stage of development, because the most corticotrophic form (di-acetylated α -MSH; Balm *et al.* 1995) was not present in these larvae. In addition to ACTH and α -MSH, other neuroendocrine factors, such as acetylcholine and atrial natriuretic peptide (Kloas *et al.* 1994), may be involved because, as in mammals and other teleosts, the control of cortisol release in carp is multifactorial.

Cortisol plays an important role during the early development of fish. Hwang & Wu (1993) showed that larval tilapia 1 day after hatching increased their whole-body cortisol contents when transferred to 20% seawater, indicating that at that stage the hormone may be involved in seawater adaptation. In these experiments the survival of cortisol-fed tilapia larvae after transfer from freshwater to 27.5% seawater was significantly higher than that of control larvae. Furthermore Mathiyalagan *et al.* (1996) showed that immersion of 1-day-old tilapia larvae in a cortisol solution significantly enhanced their growth, while higher concentrations caused retarded growth. Cortisol is an important osmoregulatory hormone for ion regulation in freshwater as well as in seawater fish. An important target for this action are the chloride cells of the gills. Proliferation, differentiation and Na^+/K^+ -ATPase activity of these cells are stimulated by cortisol (McCormick 1995). Li *et al.* (1995) showed that the gills of tilapia already have functional chloride cells immediately after hatching. Thus cortisol may already control chloride cell function immediately after hatching. Therefore, in view of the effects of cortisol demonstrated in these early stages, we suggest that transient activation of interrenal cortisol production as the result of stressful stimuli will have major consequences for further development of the fish.

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