Adrenocorticotropic Hormone in Relation to Interrenal Function during Stress in Tilapia (Oreochromis mossambicus)

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This study examines ACTH-like immunoreactivity in the pituitary pars distalis and pars intermedia of the freshwater teleost Oreochromis mossambicus (tilapia). Two products (tACTHα and tACTHβ) were present in both lobes. These two products also accounted for the majority of the ACTH i.r. when in vitro pars distalis incubation medium was analyzed by HPLC. In a homologous bioassay the two tilapia ACTH-like molecules and human ACTH1-39 possessed similar corticotropic potency. The peptides were quantified using a newly validated radioimmunoassay, which was also used to measure ACTH in plasma of unstressed and stressed fish. Short-term (<12 min) stress rapidly increased plasma cortisol, reaching levels of around 300 ng/ml in 5 min. Surprisingly, this initial elevation was not accompanied by a rise in plasma ACTH levels. A more prolonged (3 hr) confinement in pairs resulted in high plasma cortisol and ACTH levels in one fish of every pair. The second fish had control ACTH levels and only marginally elevated cortisol levels. Therefore, in this species social interactions seem to influence the reaction of the pituitary-interrenal axis to stress. The short-term cortisol response to disturbance could be abolished completely by pretreating fish in vivo with cortisol for 48 hr. This treatment did not alter circulating ACTH levels. It is concluded that tilapia did not rely on circulating ACTH for a rapid elevation of plasma cortisol levels. Both neuronal mechanisms and cortisol feedback may regulate the pituitary-interrenal axis at the level of the interrenal.

As in other vertebrates, in teleosts a stressor initially stimulates adrenal cortical activity (Donaldson, 1981). Consequently, elevated plasma corticosteroid levels are often considered an operational definition of stress. Cortisol, the main teleost corticosteroid produced by the interrenal cells located in the headkidney (Sangalang et al., 1972; Balm et al., 1989, for tilapia), exerts a variety of functions, including regulation of energy metabolism, ionic homeostasis, and immune competence (Donaldson, 1981; Maule et al., 1987; Barton and Iwama, 1991). Although there is no comprehensive model integrating these functions, most authors favor an adaptive function of cortisol during stress, in particular during the initial phase (Munck and Náray-Fejes-Tóth, 1992). Prolonged elevations of plasma cortisol, however, will be one of the causative factors leading to the deleterious effects of chronic stress (Barton and Iwama, 1991). This illustrates the careful balance fish have to maintain in regulating the activity of interrenal cortisol release. The stress response in fish has been studied in a variety of species, and recently strains have been selected on the basis of the cortisol response (Pottinger et al., 1992; Fevolden and Røed, 1993). Interestingly, some of these strains also differed in their immune characteristics (Fevolden and Røed, 1993). It has been reported that turkeys selected for low plasma corticosterone response to stress exhibited lower natural mortality and higher growth rate and egg production than

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high responding counterparts (Brown and Nestor, 1973). These results indicate the fundamental impact of the stress-related rise in corticosteroid output.

Although evidence is accumulating that the regulation of the interrenal cells in fish is more complex than previously assumed (Donaldson, 1981), ACTH is still considered the major corticotropic factor, basically because of its potency (Rance and Baker, 1981). ACTH is the main splicing product of the prohormone POMC in the pituitary corticotropes, both in fish (Rodrigues and Sumpter, 1983) and in higher vertebrates (Jones and Gillham, 1988). Despite the many studies on the effects of stress on cortisol output in teleosts, relatively little is known about the effects of stress on the pituitary corticotropes; this reflects a lack of sufficiently specific and/or sensitive assays for fish ACTH, in particular for nonsalmonids. Nevertheless, information on ACTH, in addition to cortisol, would further increase insight into the regulation of the stress response in fish. The cortisol response to stressors is finely tuned by many mechanisms operating at the levels of the hypothalamus, the pituitary, and interrenal. To date, evidence on these mechanisms in fish largely stems from in vitro data (Fryer et al., 1984; Bradford et al., 1992). The simultaneous measurement of plasma ACTH and plasma cortisol in experimental animals will yield valuable information on the relevance of these regulations in vivo. Furthermore, in salmonids (Sumpter et al., 1986) a rise in ACTH output precedes the stress-related cortisol response. Therefore, in some cases ACTH might be a more sensitive or even a more precise index for stress than cortisol.

An ACTH RIA for tilapia (Oreochromis mossambicus) has been developed and validated using an antiserum previously demonstrated to detect ACTH in a variety of vertebrates (Dores et al., 1993; R. M. Dores, personal communication). This assay was used to characterize ACTH-related material in the pituitary (neurointermediate lobe (NIL) and pars distalis (PD)) and in incubation medium by HPLC and subsequent bioassay. To investigate the role of ACTH in stress-related corticosteroidogenesis in this species, two stress protocols were tested: capture by netting (minutes) and capture + subsequent confinement in pairs (3 hr). Under these conditions, plasma cortisol, ACTH, and glucose were measured. Finally, aspects of the regulation of the corticotropes, including cortisol feedback, were investigated in vivo and in vitro.

MATERIALS AND METHODS

Mature male tilapia (O. mossambicus) from our laboratory stock were kept in groups of nine in 120-liter all-glass aquaria filled with artificial freshwater under a 12L/12D light regime. The fish, weighing 18 ± 2 g (n = 205), were fed tropical fish food (Tetramin). The response to a short-term handling was investigated by netting fish one by one from aquaria at regular intervals. This procedure took 12 min, during which seven fish were sampled. Nine aquaria were sampled this way, which yielded nine fish captured as “firsts,” nine fish captured as “seconds,” etc. Immediately upon removal from the aquaria, 200 p.l blood was collected from the caudal vessels in EDTA/aprotinin; 1.5 mg Na₂EDTA/3000 KIU aprotinin (Serva GmbH, Heidelberg, Germany). No anesthesia was applied; the fish were killed by spinal transection. Plasma was obtained by centrifugation (3 min, 10,000#), aliquoted, and stored at -20°. In each aquarium, the two remaining fish were confined in a small net for 3 hr and then sampled as described. To study the effect of steroid feedback on the capture response and in vitro cortisol release, fish were pretreated for 48 hr with cortisol delivered via the food. Briefly, Tetramin flakes were sprayed with cortisol dissolved in ethanol, which was evaporated. Control food was treated with ethanol only. Eight groups of fish (n = 7 per aquarium) were either given cortisol-containing meals (four groups) or given control meals (four groups) at t = -48 hr and t = -24 hr prior to sampling. Each meal was 1% of total body weight; total dose per 48 hr was 20 mg cortisol/kg body wt. Sampling took 9 min per aquarium.

Assays

Cortisol was measured by radioimmunoassay using a commercial antiserum (Steranti Res. Ltd., UK). The procedure of the RIA using this antiserum (validated previously for the measurement of cortisol in fish plasma; Pickering et al., 1987) was slightly modified.
Briefly, 5 µl standards or unknowns were incubated overnight with 100 µl tritiated cortisol (4000 dpm; Amersham Nederland BV, 's Hertogenbosch, The Netherlands) and 50 µl antiserum (diluted to yield one-third of the titer recommended by the suppliers). All constituents were in phosphate buffer (0.01 M, Sigma P 4411; Sigma Chemie, Bornem, Belgium) containing 0.1% (w/v) BSA (Fluka 05475, Fluka Chemie AG, Buchs, Switzerland). Free and bound hormone were separated by adding 1 ml of ice-cold dextran-coated charcoal to each tube containing 2 mg Norit A (Fisher Scientific Company, Fair Lawn, New Jersey) and 0.2 mg dextran (70 kDa, Fluka 31390, Fluka Chemie AG). The tubes were centrifuged at 4° (1600 x g, 10 min in a Jouan CR 412). 800 µl supernatant was mixed with 4 ml Aqualuma scintillation fluid, and counted in a Pharmacia Wallac 1410 liquid scintillation counter. The sensitivity of this assay was 1.6 pg/tube, and 25 pg cortisol half maximally displaced the labeled hormone from the antibody. The intra- and interassay coefficients of variation were 7.9 and 8.5%, respectively.

The steroid was measured in unextracted samples; routinely, tilapia plasma samples were diluted 10 times in assay buffer.

Tilapia ACTH was measured by RIA using an antiserum raised against ACTH1-24 kindly donated by Professor R. M. Dores (see Dores et al., 1993). Routinely, 25 µl standards (NISS, London, UK; hACTH1-39) or unknowns were incubated at 4° with 50 µl antiserum (1:7000) and 100 µl assay buffer (63 mM Na2HPO4, 13 mM Na2EDTA, 0.02% (w/v) NaN3) containing 25,000 KIU aprotinin (Bayer), 100 µl Triton X-100 (Sigma), and 250 mg BSA ORHD 20/21 (Hoechst) per 100 ml. After 72 hr, 4500 cpm iodinated hACTH1-39 (2000 Ci/mmol; Amersham Nederland BV, 's Hertogenbosch, The Netherlands) and 50 µl antiserum (diluted to yield one-third of the titer) was added to the tubes, which were incubated for another 24 hr at 4°. Second antibody (100 µl; SAR diluted 10 times in a 0.01% (w/v) rabbit IgG—Sigma I 5006—solution) was then added to the tubes, which were incubated 20 min at 21°. The second antibody was donated by Professor Benraad, Department of Clinical Endocrinology, University of Nijmegen, The Netherlands. Finally, 1 ml of an ice-cold PEG solution (7.5% w/v PEG 6000; Merck, Darmstadt, Germany) in assay buffer (from which aprotinin and BSA had been omitted) was added, and the tubes were centrifuged (10 min, 1500g, Jouan CR 412). The supernatants were decanted, and the pellets were counted in a LKB Wallac Clinigamma.

Pituitary and PD homogenates were prepared in all-class homogenizers in 0.01 M HCl, centrifuged (30 min, 10,000g), and the supernatants were diluted in assay buffer. For the displacement curves only, RIA sample volume was 50 µl.

α-MSH immunoreactivity was measured by RIA following van Zoest et al. (1989). Plasma glucose was measured in deproteinized samples using a commercial kit (Boehringer-Mannheim, Germany).

HPLC

Reverse-phase high-performance liquid chromatography was performed as described by Martens et al. (1982). To the fractions collected, 10 µg BSA was added, and the samples were dried in a Speedvac (Savant). Peptides were dissolved in 0.1 N HCl/MeOH (1:1), and ACTH and α-MSH immunoreactivities were measured by RIA.

Superfusion

Superfusions of headkidney or pituitary rostral pars distalis tissues were performed as described previously (Balm, 1986) with the exception of the flow rate during the headkidney incubations (75 µl/min). Headkidney tissue was pulsed with dried (Speedvac) HPLC fractions or hACTH1-39 (Peninsula Laboratories, England) in superfusion medium. Pars distalis tissue was challenged for 10 min with a hypothalamic extract (HE; 2 hypothalami prepared in 0.01 M HCl, and subsequently diluted 100 times in superfusion medium), ovine CRH (10 min, 0.5 nM; Bachem, Bubendorf, Switzerland), or a high K+ pulse (5 min, 50 mM). The maximally stimulated release rates were tested against the effect of a saline pulse (to overcome the increase in spontaneous ACTH release; see Results) and were expressed as percentage of prepulse values. Prepulse release rates were 3.6 ± 0.5 pg/min/PD (HE experiment, n = 10), 3.4 ± 0.4 pg/min/PD (CRH experiment, n = 12), and 4.9 ± 0.6 pg/min/PD (K+ experiment, n = 30). The saline values from the three experiments were pooled, yielding n = 26. The pulses were given at t = 220 min.

Statistics

All data are presented as average ± SEM. To test differences between experimental groups, data were analyzed by the Mann-Whitney U test; P < 0.05 was accepted as a significant level of difference.

RESULTS

Serial dilutions of plasma from stressed tilapia and pituitary homogenates displaced radiolabeled ACTH from the antibody in parallel with dilutions of the standard used (Fig. 1). The assay procedure produced a very reproducible standard curve and could measure ACTH levels in plasma samples (25 µl) from unstressed animals: 47 ± 4 pg/ml (n = 25). The detection limit and the ED50 were 0.32 and 4.3 pg per tube, respectively; intra- and interassay coefficients of variation were 6.2 and 7.2%, respectively.
The cross-reactivity with monoacetylated α-MSH was negligible. Three other antibodies were tested: one donated by the NIDDK (previously demonstrated to cross-react with goldfish ACTH-like molecules; Tran et al., 1989), and an antibody directed against the N-terminal portion of the ACTH molecule ("N-term," donated by Professor Sumpter; Sumpter, 1986), and an antibody from the IgG Corporation (Nashville, TN), which is highly sensitive and has been used to measure salmonid ACTH by RIA (Sumpter and Donaldson, 1986; Balm and Pottinger, 1993). These antibodies did not satisfactorily detect tilapia ACTH-like material in our RIA system, assessed from the lower sensitivity (NIDDK, N-term), or poor displacement curves with serial dilutions of tilapia pars distales homogenates (NIDDK, IgG; not shown). Conversely, the present antibody could not measure rainbow trout ACTH in plasma as judged by the poor displacement of radiolabeled ACTH by dilutions of plasma from stressed trout (Fig. 1).

HPLC analysis of the ACTH immunoreactivity in tilapia pituitary homogenates revealed the presence of two products in both the PD and the NIL, which eluted from the column prior to the hACTH$_{1-39}$ standard (Fig. 2). The ratio between the peak areas of the two ACTH-IR peaks was similar in both lobes, but the pars distalis contained approximately eight times more ACTH immunoreactivity than the NIL. The more apolar ACTH product ("A") coeluted with monoacetylated α-MSH. Although the majority of the α-MSH immunoreactivity was recovered from the NIL homogenate, significant amounts of desacetylated-α-MSH in particular were present in the PD. The relative amounts of the three α-MSH prod-
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Fig. 2. HPLC immunograms of tilapia pars distalis and neurointermediate lobe homogenates (●). The open circles illustrate the elution profiles of ACTH and α-MSH standards. Please note the different Y axes in the two α-MSH panels. PD and NIL lobes from two fish were homogenized and subjected to HPLC. Between 10- and 25-min 0.3-min fractions were collected; 0.5-min fractions were collected between 25 and 45 min. The major α-MSH immunoreactive peaks in this system have been demonstrated previously to represent tilapia des-, mono-, and diacetylated α-MSH (Lamers et al., 1991).

When tested in a homologous bioassay, both HPLC purified immunoreactive ACTH products were corticotrophic (Fig. 3). Apparently, at the concentration tested (2500 pg ACTH-IR/ml), both tilapia products evoked similar responses as the human standard.

During in vitro incubation, tilapia corticotropes released both ACTH products (Fig. 4a; A and B). The ratio between the amounts of both ACTH forms was similar to that in the lobe. In addition, a minor fraction of total medium ACTH immunoreactivity was recovered in several smaller peaks. Initially, the NIL and the PD released equivalent amounts of ACTH (Fig. 4b), but with time the melanotropes did not maintain this release rate, and from 80 min...
in vitro onwards the PD released significantly more ACTH than the NIL (P = 0.014). At $t = 340$ min, the release ratio between the PD and the NIL was 11.7 ± 3.4 (Fig. 4; $n = 4$). In vitro α-MSH release by the two lobes (not shown) gave the opposite picture, with the PD releasing undetectable amounts of α-MSH. The amount of ACTH released by the NIL during the initial 80 min approximated the ACTH content of a fresh NIL (Fig. 1). The in vitro release of ACTH by the PD could be stimulated by a hypothalamic extract, CRH, and K⁺ depolarization (Fig. 4c).

Tilapia appeared very sensitive to sequential sampling from groups judged by the extremely fast cortisol response (Fig. 5). At none of the sample points was the rise in plasma cortisol values ($P < 0.001$ compared with the animals caught first) accompanied by significant rises in either ACTH or glucose in plasma. The discrepancy between ACTH and cortisol levels was most notable in the fish caught second. From each of the nine groups sampled, the last two remaining fish were confined in their home aquaria for 3 hr. After this period, a marked dichotomy in cortisol levels was observed (Fig. 5). In every pair, one fish had control or moderately elevated cortisol levels, whereas the other fish exhibited levels of several hundred ng/ml. This difference was not related to the sequence in which the fish were sampled from each pair (30 sec in between the fish from each pair). When averaged separately, the cortisol levels in low responders (L) were lower than those in the fish captured just prior to the confinement period, but were twice those of fish captured as firsts. The high responders (H), however, maintained extremely high cortisol levels, which were significantly higher than those in the fish captured just prior to the confinement period ($P = 0.027$). The high significant difference in cortisol levels between the high

Fig. 3. Corticotropic activity of tilapia ACTH immunoreactive products. Peak HPLC fractions from a pars distalis homogenate and a ACTH standard run were dried and dissolved in superfusion medium to yield a concentration of 2500 pg/ml. Headkidneys from control fish were challenged with these solutions for 20 min. Results presented as increase in cortisol release over baseline release. Baseline cortisol release was $1.6 \pm 0.4$ pg/min/g (tACTHA; $n = 12$), $1.5 \pm 0.5$ pg/min/g (tACTHB; $n = 12$), and $1.1 \pm 0.4$ pg/min/g (hACTH₁₋₃₉; $n = 8$).
Fig. 4. *In vitro* ACTH release by tilapia PD lobes. For the ACTH HPLC immunogram (a), two PDs were incubated for 1.5 hr in 1 ml of incubation medium, which was subsequently subjected to HPLC. To assess the unstimulated *in vitro* ACTH release rates (b), four PDs and four NILs were superfused individually for 340 min. The effect of a hypothalamic extract (HE), CRH, and K+ depolarization was investigated using PDs in superfusion (c).

The cortisol response to the capture procedure could be abolished completely by *in vivo* cortisol treatment (Fig. 6a): none of the cortisol values of the cortisol-treated fish were different from the value in the fish caught first. In the control groups, there was a significant capture sequence effect which was evident from the fish caught second onwards (*P* = 0.014). Cortisol levels in cortisol-treated animals caught first were significantly higher than their control counterparts (*P* = 0.029). Despite the different cortisol response to the capture protocol in the experimental groups, their plasma ACTH levels were similar: 55 ± 9 versus 59

11 pg/ml for controls (*n* = 27) and cortisol-treated fish (*n* = 28), respectively. The values from the two groups were pooled because there was no fish number effect on plasma ACTH in either group. Two other parameters were affected by cortisol treatment: plasma glucose [68.9 ± 5.2 versus 122.1 ± 8.2 mg/100 ml for the controls (*n* = 28) and for cortisol-treated fish (*n* = 28), respectively; *P* < 0.001] and *in vitro* cortisol release (Fig. 6b). To limit the possible influence of the capture sequence on this parameter, only the headkidneys from the first two fish from each groups were superfused *in vitro*. The spontaneous cortisol release in the cortisol-treated group was lower than in controls (*P* = 0.014 during the first fraction collected). This difference persisted during the entire incubation, although both curves seemed to have similar baselines.

**DISCUSSION**

The present study demonstrates the presence of two major ACTH immunoreactive products in the corticotropes and the melanotropes of tilapia (*O. mossambicus*). Both forms are released *in vitro* into the medium, in particular by the corticotropes.

and low responders also corresponds to differences in the ACTH levels of the same individuals. In this case, only the ACTH levels in the high responders were significantly higher than those of the unstressed fish ("firsts"). In contrast to the divergence in the cortisol and ACTH responses to confinement, both groups were equally hyperglycemic.
In contrast to the situation in higher vertebrates and in salmonids (Sumpter et al., 1986), the cortisol response to handling was not preceded by a rise in plasma ACTH. A prolonged stress treatment, however, (confinement in pairs) resulted in both cortisol and ACTH responses. However, this was evident in only one fish of every pair, pos-
plasma cortisol (ng.ml\(^{-1}\))

\[\begin{array}{cccccc}
240 & 200 & 160 & 120 & 80 & 40 \\
1 & 2 & 3 & 4 & 5 & 6 & 7 \\
\end{array}\]

\(\text{in vitro cortisol release (pg.miri'1.g'1)}\)

\[\begin{array}{cccccc}
20 & 16 & 12 & 8 & 4 & 0 \\
0 & 120 & 240 & t (min) & & \\
\end{array}\]

Fig. 6. The effects of cortisol administration on the reaction of the pituitary-interrenal axis to a capture protocol (a). (b) The in vitro cortisol release by the headkidneys of the experimental fish (n = 8 per group; see text for details). Closed and open circles refer to control and cortisol-treated fish, respectively.

possibly indicating a social interaction in the regulation of the pituitary-interrenal axis during confinement stress in this species.

There are several reasons why the two immunoreactive products identified by IPILC are concluded to represent tilapia ACTH. First, the antiserum used has been demonstrated to detect ACTH molecules in variety of (nonmammalian) vertebrate species (Dores et al., 1993; R. M. Dores, personal communication). Second, both products were bioactive in a homologous bioassay. The finding that the bioactivity was comparable with that of the hACTH\(_{1-39}\) supports our identification since none of the other POMC-derived peptides identified to date in other vertebrates possess corticotropin activity comparable to ACTH (Lowry, 1984; Rance and Baker, 1981). For this reason the small contamination of the α-MSH HPLC fraction with monoacetylated α-MSH (5% maximally) may be assumed not to interfere in the bioassay. Prolactin, the only major non-POMC factor in the tilapia rostral pars distalis, elutes at 46 and 50 min (tPRL\(_{177}\) and tPRL\(_{188}\), respectively) in the HPLC system used (Balm, unpublished). Third, the different results obtained with tilapia PD and NIL tissue confirm the characteristic picture of POMC processing in these two pituitary lobes. Finally, the release of ACTH immunoreactivity could be markedly stimulated with CRH.

The ACTH radioimmunoassay described combined sensitivity with specificity for tilapia ACTH-like molecules in particular, and could be used to measure ACTH immunoreactivity in tilapia, but not in trout plasma. It appears that the species specificity between fish ACTH molecules is considerable because for three species studied (goldfish, Tran et al., 1989; salmonids, Sumpter, 1986; tilapia, this study) three dif-
different antibodies have been used which were demonstrated not to be interchangeable (Sumpter, 1986; this study). The ACTH antibody used possesses 10% cross-reactivity with CLIP (Dores et al., 1993). Therefore, on the basis of the amount of α-MSH in the NIL homogenate, a major (third) peak in the NIL ACTH immunogram was anticipated. Its absence indicates that the antibody does not detect tilapia CLIP. Comparison of the present data with those obtained with other species confirms the species specificity: two major immunoreactive products in tilapia (see Tran et al., 1989, for goldfish), which appear identical in PD and NIL (see Sumpter, 1986, for chinook salmon), and which both possess a ratio between immunoreactivity and bioactivity of around 1 (see Scott and Baker, 1975, for rainbow trout). Therefore, the tilapia data in particular comply reasonably well with the characteristic vertebrate picture (Jones and Gillham, 1988). It seems unlikely that these discrepancies between fish can be explained solely by the different methods (separation, assays) employed, and therefore indicate species-specific corticotrope functions. At present, the nature of the different forms in tilapia is unclear. The fact that both tilapia forms are released into the medium might indicate differential processing of POMC in the corticotropes of this species, possibly leading to phosphorylated and nonphosphorylated forms (Bennett et al., 1983) or to molecules of varying size (e.g., by carboxypeptidase activity; Friedman et al., 1993). The possibility that one of the forms represents POMC can be excluded, since the prohormone elutes at 41 min in the HPLC system used (Martens et al., 1982). Different degrees of glycosylation appear unlikely on the basis of the data by Iturriza and Estivariz (1986).

The α-MSH immunoreactivity present in the tilapia corticotropes indicates processing of ACTH in PD POMC cells of this species. The presence of α-MSH did not result from contamination of the PD with NIL tissue or of residual NIL α-MSH on the HPLC column, since the ratio between the three α-MSH forms was different between the two tissues. The processing of ACTH into desacetylated α-MSH in particular has also been demonstrated in the corticotropes of other vertebrates (Tanaka and Kurosomi, 1986; Dores et al., 1993). A function of this process is unclear, but it has been observed to be most prominent during fetal/neonatal developmental stages (Dores et al., 1993) and to be under regulation by CRH (Iturriza and Ferese-Spinelli, 1991).

A surprising finding was the increase in unstimulated ACTH release by tilapia PD tissue with time in vitro since these data are in conflict with in vitro data of Tran et al. (1989) on goldfish. However, Ball et al. (1972), studying the corticotropes of several fish species during in vitro culture, also reported an apparent activation of eel and rainbow trout ACTH cells under these conditions. This was taken to indicate a species-specific regulation of corticotrope function. The present data may indicate an overriding inhibitory control of basal secretory activity of tilapia corticotropes in vivo. At present the additional peaks in the HPLC immunogram of the incubation medium are unknown. They might either result from release-associated modifications of one or both of the major products or arise in the medium (e.g., sulfoxide forms of ACTH). However, only a small fraction of the total immunoreactivity was recovered in these peaks. These products are concluded to be of minor importance because the high steroidogenic potency of the two major ACTH-like products (tACTH_A and tACTH_B), probably precludes, a major biological role for other products. In addition to the differences in both ACTH and α-MSH release between the NIL and the PD POMC-producing cells, it also appeared that the pattern of unstimulated ACTH release by the melanotropes differs significantly from that of unstimulated α-MSH release by these cells (Lamers et al., 1991).
The differences in PD and NIL ACTH content and release suggest that the PD is the major source of circulating ACTH in tilapia. A similar conclusion was drawn for trout by Rodrigues and Sumpter (1983).

The almost immediate cortisol response to handling in *O. mossambicus* corroborates recent results of Foo and Lam (1993) on the same species. Although these authors also sampled their fish by netting them one by one, they did not observe the fish number effect reported here, possibly because of the additional anesthetization step used by these authors. The promptness of the response in tilapia is illustrated by comparison with that in brown trout to a similar handling protocol (Pickering et al., 1982). The data of Sumpter et al. (1986) on rainbow trout also indicate that plasma cortisol levels did not react to handling and confinement within 8 min. Also, in humans cortisol responses become manifest after 10 min (Hermus et al., 1984). In addition to the rapid onset of the cortisol response to disturbance, a second notable feature of the response in tilapia is the ability of (a subset of) the animals to rapidly lower their plasma cortisol levels in the continuous presence of the stressor. An efficient cortisol clearance mechanism probably plays an important role in this process. Foo and Lam (1993) demonstrated that during the first hour after a stress treatment plasma cortisol in *O. mossambicus* had dropped from 144 ng/ml to control levels (5 ng/ml). Previous results on the effects of cortisol treatment on plasma cortisol levels (Lam, 1986) corroborate the short half-life of cortisol in tilapia plasma suggested by the data of Foo and Lam. The ability of tilapia to quickly downregulate the activity of the pituitary interrenal axis will be of obvious advantage to the fish, since in this way they are able to benefit from the alarm reaction of interrenal activation, but can avoid the deleterious consequences of prolonged elevations in plasma cortisol (Barn and Iwama, 1991).

A unique feature of the initial interrenal response in tilapia subjected to handling is its apparent ACTH independence. In some of the groups studied, individual fish had elevated plasma ACTH levels. However, there was no correlation between plasma cortisol and plasma ACTH ($r = 0.12, P > 0.05, n = 63$), which further supports the conclusion that during the first minutes ACTH and cortisol are independently regulated. The response to confinement in particular in the high responders, however, indicates that a more prolonged activation of interrenal cortisol output might rely on elevated ACTH levels. The divergence in the interrenal response to confinement ties in with reports on eel (Peters et al., 1981) and rainbow trout (Pottinger and Pickering, 1992), in which fish were also exposed to confinement in pairs. The present data extend this difference to the pituitary level. The different response in pairs usually is discussed in terms of hierarchy, the low responders being dominant. Tilapia species are hierarchal (de Oliveira Fernendez and Volpato, 1993), and therefore we suggest that in our fish differences in hierarchal status (established prior to or during the confinement period) underly the different stress responses. As suggested for other species, this phenomenon might selectively predispose high responders to infectious diseases (Maule et al., 1987), as well as reduce their development and growth rate. Other relevant features include the facts that subordinates generally suffer most from additional stress conditions (Schreck, 1981) and social rank in some fish is associated with interrenal mass (Erickson, 1967) and interrenal cell activity (Noakes and Leatherland, 1977; Schreck, 1981). Whether differences in HPA axis activity associated with social status are also expressed anatomically at levels other than the interrenal is unknown. Social regulation of the brain–pituitary–gonadal axis in *Haplochromis burtoni* (an African cichlid fish) occurs through reversible modulation of
soma size changes in a population of GnRH neurons in the ventral forebrain, territorial males having larger cell bodies (Francis et al., 1993).

The results of the cortisol administration experiment confirm the apparent ACTH independence of the initial cortisol response since cortisol treatment abolished the cortisol response to capture but did not affect plasma ACTH levels. The data further suggest that tilapia corticotropes are relatively insensitive to direct corticosteroid feedback, which may seem surprising in view of previous in vivo and in vitro results in other fish (Pickering et al., 1987, Olivereau and Olivereau, 1989). However, Fryer and Peter (1977) demonstrated that implanting cortisol-releasing pellets adjacent to the NLT or NPO of the goldfish hypothalamus, but not in the pituitary, inhibits the cortisol response to handling (saline injection). They concluded that in their species, cortisol mainly acted at the level of the hypothalamus to inhibit the reaction of the corticotropes to handling. One conclusion that can be drawn from the present data is that the plasma cortisol level is not a reliable index for corticotrope activity (Fryer and Peter, 1977) because the results demonstrate that plasma cortisol levels may be elevated in the absence of corticotrope activation. Two mechanisms might explain this cortisol-induced inhibition of the interrenal stress response in tilapia, which apparently bypassed the ACTH cells. First, direct cortisol feedback at the level of the interrenal cells: our in vitro data might support this notion. Assuming that the initial in vitro cortisol release reflects in vivo secretory rates (Balm, 1986; Balm and Pottinger, 1993), the data demonstrate that interrenal cells of cortisol-treated tilapia release less cortisol than in controls, despite similar plasma ACTH levels. This would imply that ACTH is of minor importance for the maintenance of basal in vivo cortisol output. Cortisol has recently been suggested on the basis of in vitro experimental evi-

dence to act at the level of the interrenal cells to render them less sensitive to ACTH (Bradford et al., 1992). However, this mechanism cannot explain the suppression of the interrenal response to capture by exogenous cortisol, since that initial response does not rely on ACTH. Alternatively, the results would be consistent with the idea that the initial phase of the interrenal response is regulated by neuronal mechanisms, which are sensitive to cortisol, either at the level of the CNS or the interrenal cells. The kinetics of the cortisol response seem to favor this type of regulation. Furthermore, corticosteroidogenesis is modulated by a number of neurotransmitters delivered from nerve endings (Charlton, 1990) or released from nonsteroidal adrenal cells, regulating steroidogenesis in a paracrine fashion.

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