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Abstract

This work describes the application of a homologous radioimmunoassay (RIA) in the measurement of plasma and pituitary growth hormone levels of tilapia maintained at 20 and 26°C, using recombinant tilapia growth hormone (rtiGH). RIA sensitivity (ED_{90}) was 0.08 ng/ml and ED_{50} was 0.62 ± 0.02 ng/ml. Intra- and inter-assay coefficients of variation were 3.3 and 10.6% respectively, for a plasma sample of 1 ng/ml of tiGH. Mammalian or salmonids growth hormones, thyrotropins or tilapia prolactins did not show cross-reactivity with rtiGH antiserum. Plasma and pituitary extract of *Oreochromis niloticus* gave inhibition curves parallel to the rtiGH standard curve without interaction with serial dilutions either of tissue extracts or plasma from hypophysectomized tilapia. Pituitary extracts from other cichlid fish showed parallel competitive binding curves. Pituitary extracts of perciform fish exhibited a lower affinity to anti-rtiGH serum, while extracts of species from more distant families showed only negligible cross-reactivity or none at all. The effects of water temperature on plasma concentrations and pituitary contents of GH were investigated in tilapia acclimatized to 26 and to 20°C. Measurements have been performed at the beginning and at the end of a two-week experiment. Whereas in fish from colder water plasma GH mean values were decreased, only a similar tendency was observed in pituitary GH contents. These results emphasize the hypothesis wich reports that the increase of growth with temperature is, at least in part, controlled by GH.

Keywords: Cichlids, tilapia, growth hormone, pituitary hormone, plasma, radioimmunoassay, water temperature.

Application d'un dosage radio-immunologique de l'hormone de croissance recombinée de cichlidés, à la mesure de l'hormone de croissance GH endogène chez le tilapia (Oreochromis niloticus) *maintenu à différentes températures.*

Résumé

Ce travail décrit l'application à la mesure des niveaux plasmatique et hypophysaire de l'hormone de croissance (GH), chez des tilapias maintenus à 20 et à 26°C, d'un dosage radio-immunologique homologue (RIA), utilisant l'hormone de croissance recombinées de tilapia (rtiGH). La sensibilité du dosage (ED_{90}) est de 0,08 ng/ml et l'ED_{50} de 0,62 ± 0,02 ng/ml. Les coefficients de variation intra- et inter-dosages sont respectivement de 3,3 et 10,6 %, pour un échantillon de plasma de tilapia titrant 1 ng/ml d'hormone de croissance (tiGH). Ni les préparations de GH ou d'hormone thyréotrope de mammifères ou de salmonidés, ni celles de prolactines de tilapia ne provoquent de réactions croisées avec notre immum-sérum de GH recombinées de tilapia. Le plasma et les extraits hypophysaires d'*Oreochromis niloticus* procurent des courbes d'inhibition parallèles à la courbe standard de rtiGH, sans interaction avec des dilutions sériées d'extraits tissulaires ou de plasma de tilapias hypophysectomisés. Les extraits hypophysaires de perciformes présentent une moins grande affinité pour l'immum-sérum, ceux d'espèces de familles plus éloignées ne

présentent que peu ou pas du tout de réactions croisées. Les effets de la température de l'eau sur les niveaux en hormone de croissance des plasma ou des contenus hypophysaires, ont été étudiés chez des tilapias acclimatés à 26 et à 20°C. Les mesures ont été réalisées au début et à la fin d'une expérimentation de 2 semaines. Tandis que chez les poissons en eau plus froide, les teneurs moyennes de GH décroissaient, une tendance dans ce sens, a seulement pu être observée sur leurs contenus hypophysaires. Ces résultats renforcent l'hypothèse selon laquelle la croissance liée à l'augmentation de température, est réalisée sous le contrôle de GH.

Mots-clés : Cichlidés, tilapia, hormone de croissance, hormone hypophysaire, plasma, dosage radio-immunologique, température de l'eau.

INTRODUCTION

A revival of the interest in tilapias has been noted in the last ten years, in Asiatic and African countries. At the present time, production of tilapias has reached third in the world (Lazard, 1990), as compared with production of other fish. With already more than 20 cultured species (Guerero, 1982), tilapias belong to the Cichlidae, genera *Oreochromis, Sarotherodon* and *Tilapia*, which are particularly appreciated in fish farming for their robustness, their relatively fast growth rate, their ease of reproduction and their wide distribution.

It has been observed that tilapias are tolerant to wide temperature ranges (Philippart *et al.*, 1982; Chervinski, 1982) and this factor is known to influence their growth rate (Caulton, 1982; Rana, 1990). Environmental variables such as temperature and photoperiod undoubtedly contribute to seasonal variations in growth rates. Nevertheless, the specific effect of the temperature, dissociated from the photoperiod, has rarely been studied (Marchant *et al.*, 1986, in *Carassius auratus* ; Barett *et al.*, 1989, in *Salmo gairdneri*; Björnsson *et al.*, 1989; Stefansson *et al.*, 1991; Björnsson *et al.*, 1994, in *Salmo, salar*).

Furthermore, it has been well established that growth hormone (GH) isolated from a variety of species is growth promoting in teleost fish (Donaldson *et al.*, 1979; Le Bail *et al.*, 1993*b*). The influence of growth hormone on fish growth has been revealed to be dependent upon rearing temperature (Weatherley and Gill, 1987). After Adelman (1977, 1978) studied the influence of four levels of temperature on the growth stimulating ability of bGH in carp, he hypothesized a decrease of endogenous growth hormone production, at below- or above-optimal growth temperature, resulting in an increased effectivness of exogenous GH injections in carp. Experiments on trout have demonstrated that recombinant trout GH was able to sustain positive anabolism at temperatures (1°C) that totally inhibit the growth of untreated rainbow trout (Drot *et al.*, 1990). It has been mentioned that the increase in growth rate, due to exogenous GH, was inversely proportional to temperature, in coho salmon and trout (Le Bail *et al.*, 1993*b*). Thus, it would be interesting to ascertain the variations in GH storage and circulating levels, in order to estimate its role in temperature controlled growth. It seemed possible that, partially *via* the rate of GH secretion, temperature exercises its effects on tilapia growth.

Nevertheless, the mode of action of GH and the nature of the mechanisms controlling its secretion have not been fully investigated, mainly because of a scarcity of suitable fish GH assays. Homologous radioimmunoassays (RIAs) to estimate GH circulating levels and pituitary contents in several fish species are indeed available, yet they did not appear to be suitable in getting valid measurements of immunoreactive GH in all fish groups, on account of the immunological diversity of GHs within the superorder Teleostei (Kawauchi *et al.*, 1990).

Two RIAs have already been developed for tilapia GH, using purified hormone, (Farmer *et al.*, 1976; Ayson *et al.*, 1993), in which tilapia prolactins particularly, showed cross-reactions. Thus we chose to develop a sensitive and more specific tilapia growth hormone RIA using rtiGH, wich was free from other pituitary hormones (Rentier-Delrue *et al.*, 1989*b*). A homologous RIA for the measurement of plasma levels, and pituitary contents of GH in *Oreochromis niloticus* is described in the present paper. Its fish family specificity is reported. An evaluation of plasma and pituitary GH levels was carried out at 20 and 26°C, the temperatures at which they were held for two weeks.

MATERIALS AND METHODS

Sources of hormones

Human (hGH) and bovine GH (bGH) were supplied by the National Institute of Health (NIH), Bethesda, Maryland, USA. Chinook salmon gonadotropin (sGtH) and thyroid stimulating hormone (sTSH) were provided by Dr. B. Breton INRA, Rennes, France. Recombinant tilapia GH (rtiGH), prolactins I and II ($rtiPRL_1$-$rtiPRL_2$) and recombinant trout GH (rtGH) produced according to Rentier-Delrue *et al.* (1989*a, b*) were obtained from Eurogentec, Liège, Belgium. Chinook salmon GH (sGH) and gilthead sea bream GH (sbGH), where purified from pituitary extracts (Le Bail *et al.*, 1991, 1993*a*).

Preparation of antisera

The rtiGH was dissolved in 10 mM NaOH, diluted with 0.9% NaCl, and the mixture was emulsified in an equal volume of complete Freund's adjuvant before rabbit immunization. Anti-rtiGH was obtained from rabbits after multiple intradermal inoculations on the back with 50 μg rtiGH, every two weeks for the first month, then booster injections every month, until a satisfactory titre was reached.

Iodination of recombinant tilapia GH

The rtiGH was iodinated according to the method used for sGH (Le Bail *et al.*, 1991). Unreacted iodide was separated from the labelled hormone by gel filtration on Sephadex G25 column (PD10, Pharmacia) previously equilibrated by assay buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.05% NaN_3, 1% bovine serum albumin (BSA), pH 7.5). The amount of radioactivity bound by an excess of rtiGH antibody was high and relatively constant (90–95%) in all fifteen-drop elution fractions of rtiGH peak. Specific activity oscillated around 94 μCi/μg. Iodinated hormone (^{125}I-rtiGH) was stable for about two months when stored in glycerol (2:1) at −20°C.

Sources of plasma, pituitaries and tissues

Carp (*Cyprinus carpio*), turbot (*Psetta maxima*), rainbow trout (*Oncorhynchus mykiss*) and *Oreochromis niloticus* were reared in our experimental installations. European eel (*Anguilla anguilla*) was obtained from our facilities. Pituitaries of fish from the cichlid family (*O. mossambicus, Tilapia zillii, T. guineensis, Tylochromis jentiki, Hemichromis fasciatus, H. bimaculatus, Sarotherodon galileaeus, S. melanotheron*) and of catfish (*Chrysichthys nigrodigitatus*) were collected at the fish farms of Layo, (CRO/ORSTOM) and Bouaké (IDESSA/CIRAD), Ivory Coast. Seabass (*Dicentrarchus labrax*), red mullet (*Mullus surmuletus*), grey mullet (*Mugil cephalus*), gaper (*Serranus cabrilla*), sea pike (*Sphyraena sphyraena*), sea scorpion (*Scorpaena scrofa*), forkbeard (*Phycis phycis*) and gilthead sea bream (*Sparus aurata*) were captured off the Spanish Levant Coast.

Fish pituitaries were removed as soon as possible after fish capture and homogenized in the RIA buffer (1 ml of buffer/kg fish). The homogenates were centrifuged at 2,000 g (10 min, 4°C) and the supernatant kept frozen at −20°C until assayed.

O. niloticus hypophysectomy was performed according to the transorbital approach described by Nishioka (1980). Hypophysectomized fish were kept up to 7 days before blood sampling.

All fish blood samples were collected from the caudal vessels through heparinized syringes, just after fish capture; plasma was immediately separated by centrifugation at 3,000 g (30 min, 4°C) and kept frozen until assay.

As soon as removed, tissues from *O. niloticus* were minced and homogenized in 50 mM Tris, 10 mM $MgCl_2$ (pH 7.5) containing 0.25 mg/ml soya bean trypsin inhibitor and 1 mM PMSF (1 ml/g initial tissue weight). The entire procedure was carried out at 4°C. The homogenates were then centrifuged (3,000 g, 30 min at 4°C) and the supernatant maintained frozen before assay.

RIA procedure

RIA was performed using a double antibody method under disequilibrium conditions as previously described for sGH RIA (Le Bail *et al.*, 1991). Standard rtiGH, plasma and pituitary extracts were serially diluted with assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.05% NaN_3, 0.1% Triton X-100 and 1% BSA, pH 7.5). 100 μl of each preparation was then assayed (6 replicates) in polystyrene 5 ml assay tubes. 100 μl of rabbit anti-rtiGH serum (diluted to $1:3 \times 10^6$ in assay buffer containing 0.35% non-immune rabbit serum) were added and all the assay tubes mixtures homogenized. After 24 h incubation at room temperature, 100 μl of buffered ^{125}I-rtiGH (10,000 cpm) were added to each tube. After homogenization, samples were incubated again for 24 h. The antibody-bound hormone was then precipitated by addition of 100 μl of a 16% dilution of sheep anti-rabbit γ-globulin serum in 20 mM Tris-HCl buffer, pH 7.5, containing 6% polyethylene glycol (PEG). All assay tubes were incubated, after vortexing, for 24 h at room temperature. After addition of 3 ml of 20 mM Tris-HCl, pH 7.5, assay buffer, the tubes were centrifuged for 60 min (3,000 g, 4°C). The supernatant was then discarded and the radioactivity in the bound fraction was determined in a Packard Multi-Prias gamma counter.

The radioactivity of the non-specific binding (when specific antibody was replaced by assay buffer) was about 1.5 to 2% of the total radioactivity added; in the absence of unlabelled hormone rtiGH, the radioactivity specifically bound by the antibody (Bo/T) was about 30%.

Temperature experiment

Thirty *O. niloticus* were randomly selected from our experimental installation, kept at a constant temperature of 26°C; 10 fish among them, sampled after killing, constituted the initial group of the experiment, whereas the remaining fish were equally subdivided into 2 experimental groups of similar weights: 37±5 g; 37±6 g (mean±SD). Each fish group was isolated in a freshwater aquarium and held respectively at 20 and 26°C. Fish were fasted for the first 3 days of their adaptation to their new tanks. Afterwards the two groups were fed to satiation twice a day, for two weeks, before killing and sampling (weight, blood, pituitary).

Statistics

B/Bo values derived from serial dilutions of hormones, plasma and pituitary extracts were linearized using logit transformations. The curve slopes calculated for each set of points were compared to the rtiGH standard curve by analysis of covariance. Significance was accepted at the 5% level.

The significance of difference between means of treatments for the temperature experiment results was estimated using the rank test of Wilcoxon-Mann-Whitney.

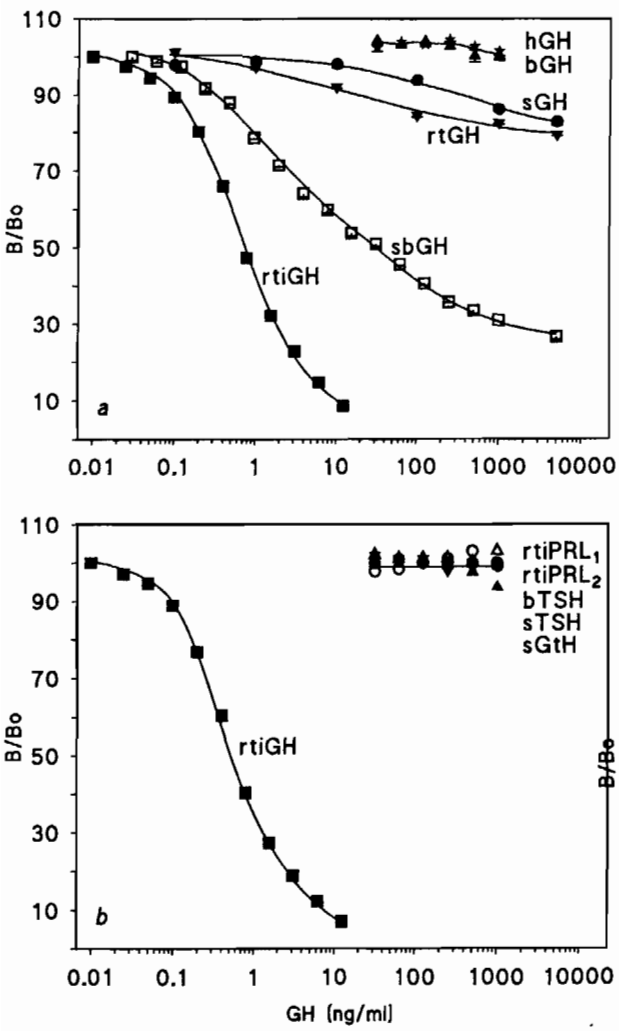

Figure 1. – Hormonal specificity: cross-reactivity of the rtiGH with mammalian and teleostean GH and pituitary hormones. (*a*) Dose response inhibition curves for recombinant tilapia Growth Hormone (rtiGH), sea bream GH (sbGH), recombinant trout GH (rtGH), chinook salmon GH (sGH), human GH (hGH) and bovine GH (bGH). (*b*) Dose response inhibition curves for recombinant tilapia GH (rtiGH), chinook salmon GtH (sGtH) and TSH (sTSH), bovine TSH (bTSH) and recombinant tilapia PRL_1 ($rtiPRL_1$) and PRL_2 ($rtiPRL_2$). Each point is the mean ± SEM of six determinations.

RESULTS

Assay characteristics

The mid-range of the RIA (ED_{50}) is 0.62 ± 0.02 ng rtiGH/ml (mean ± SEM, n = 13). 10% inhibition of maximum binding was obtained with 0.08 ng/ml. The intra-assay coefficients of variation (CVs) for three plasma samples (0.7, 1 and 1.5 ng/ml) assayed ten times in a single assay were 3.6, 3.3 and 2.9% respectively. The corresponding inter-assay CVs, determined in three separate assays, were 5.2, 10.6 and 10.3%.

None of the mammalian GH preparations (hGH, bGH) cross-reacted in the tiGH RIA (*fig.* 1 *a*). Salmonid GH (sGH, rtGH) is very slightly recognized in the assay whereas serial dilutions of sea bream GH (sbGH) exhibited a significant but not parallel displacement at concentrations 30 times higher than rtiGH (ED_{50}). *Figure* 1 *b* shows that none of the pituitary hormone preparations, such as tilapia prolactins ($rtiPRL_1$, $rtiPRL_2$) or salmon thyroid stimulating hormone (sTSH) and gonadotropin (sGtH) compete with tiGH in this assay.

Figure 2 illustrates displacement curves obtained with different tissue extracts from *O. niloticus*. Serial dilutions of pituitary extracts and plasma gave displacement curves parallel to the recombinant tilapia GH standard curve. Brain, ovary, spleen, and kidney extracts caused only very limited displacements of antibody bound labelled rtiGH at very

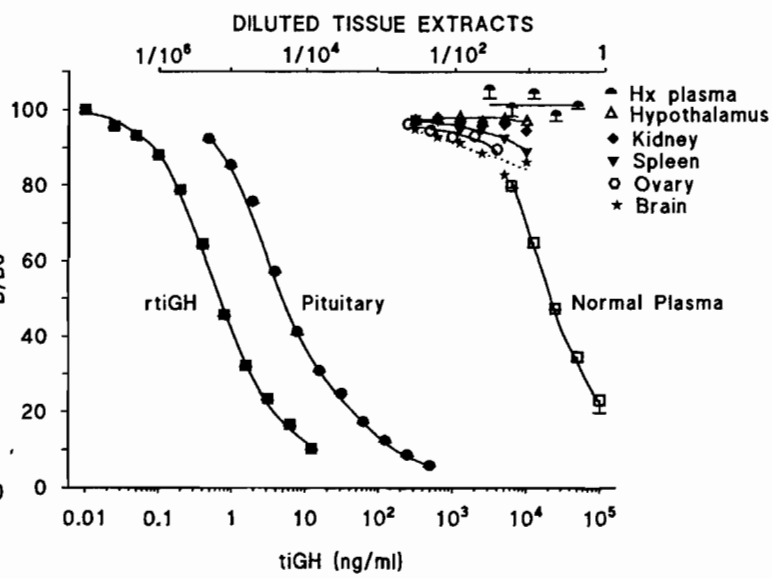

Figure 2. – Tissue specificity: displacement curves obtained with different tissue extracts from *Oreochromis niloticus*. Dose response inhibition curves for recombinant tilapia GH (rtiGH) and serial dilutions of pituitary, plasma, hypothalamus, brain, ovary, spleen and kidney extracts and a pool of hypophysectomized tilapia plasma (Hx plasma). Initial dilution of each tissue extracts: 1 ml of assay buffer for 1 Kg of body weight. Each point is the mean ± SEM of six determinations.

high concentrations. Plasma from hypophysectomized tilapia did not show any displacement. Recovery experiments were conducted by measuring tiGH added in increasing concentrations to 50 μl of plasma from hypophysectomized tilapia. The equation $y = 1.025 + 0.04$ ng/ml ($r = 0.995$) of the regression line (GH recovered *versus* GH added) did not differ significantly from 1, (data not shown). The recovery of rtiGH reached 107%.

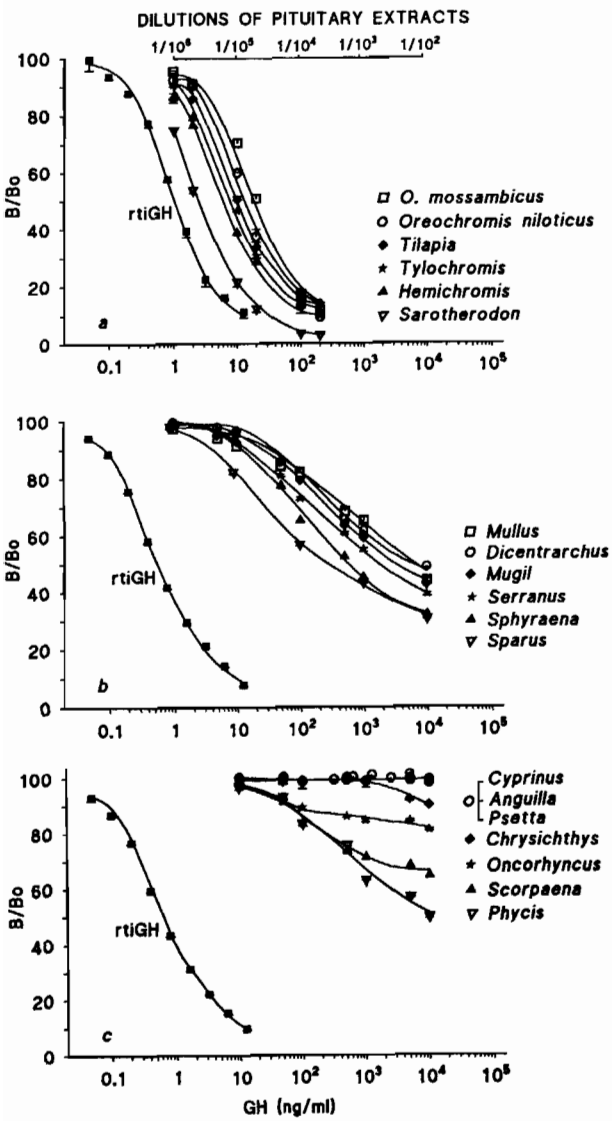

Figure 3. – Cichlids and non-cichlids immunological specificity: RIA dose-response inhibition curves of rtiGH in comparison with dilutions of pituitary extracts from different teleost fishes. Dose response inhibition curves of recombinant tilapia GH (rtiGH) and serial dilutions of pituitaries extracted from various cichlid species belonging to genera *Oreochromis, Tilapia, Tylochromis, Hemichromis* and *Sarotherodon* (*a*), various perciform species (*b*) and different distant fish orders (*c*). Initial dilution of pituitary extract: every pituitary was suspended in 1 ml of assay buffer for 1 Kg of body weight. Each point is the mean ± SEM of six determinations.

Figure 3 presents a RIA dose-response inhibition curve for recombinant tiGH in comparison with serial dilutions of pituitary extracts from several teleost fishes.

The displacement curves for pituitary extracts from different cichlid species (*fig.* 3*a*) such as *Sarotherodon, Hemichromis, Tylochromis, Tilapia* and *Oreochromis* were parallel to the rtiGH standard curve (levels of significance: 0.05). Pituitary extract dilutions from Perciform order fish: sea bream (Sparidae), seapike (Sphyraenidae), gaper and seabass (Serranidae), grey mullet (Mugilidae) and red mullet (Mullidae), exhibited significant but non-parallel displacement (*fig.* 3*b*). Pituitary extracts from forkbeard (Gadiform), sea scorpion (Scorpaeniform) and trout (Salmoniform) showed a non-parallel displacement of the antibody-bound labelled rtiGH, whereas catfish (Siluriform), turbot (Pleuronectiform), European eel (Anguilliform) and carp (Cypriniform) did not react in the assay (*fig.* 3*c*).

Temperature experiment

Results of the experiment and GH RIA on plasma and pituitary extracts from the two groups of *O. niloticus*, kept for two weeks in aquaria respectively at 20 and 26°C, are illustrated in *figure* 4. The weight of each group was significantly higher (44 ± 7 g *versus* 37 ± 5 g, $\alpha = 0.001$ at 26°C, 56 ± 7 g *versus* 37 ± 6 g, $\alpha = 0.01$ at 20°C) at the end of the experiment. At this time the lowest weights were observed in fish maintained at 20°C. The fish held at the lower temperature exhibited a lower level of plasma GH

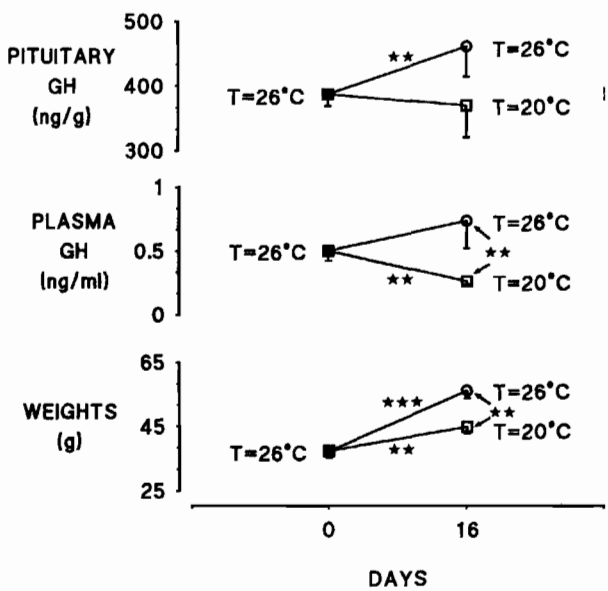

Figure 4. – Water temperature effects on GH levels in two groups of ten *Oreochromis niloticus*. Changes of the weight, plasma (ng/ml) and pituitary GH (ng/g of fish weight) of the two groups of tilapia held two weeks respectively at 20 and 26°C (Statistical significance levels: * $\alpha < 0.05$, ** $\alpha < 0.01$, *** $\alpha < 0.001$).

(0.26±0.06 ng/ml *versus* 0.74±0.56 ng/ml, $\alpha = 0.02$). Pituitary GH concentration increased in fish kept at 26°C on day 0 and day 16 (461±156 ng/g *versus* 387±65 ng/g). Nevertheless pituitary GH concentration did not show significant change in fish subjected from 26 to 20°C water (387±65 ng/g *versus* 369±155 ng/g).

DISCUSSION

The sensitivity of the assay is higher than that reported in other fish GH RIAs already developed: GH RIA in tilapia (Farmer *et al.*, 1976; Ayson *et al.*, 1993) in carp (Cook *et al.*, 1983, Fine *et al.*, 1993), in eel (Kishida and Hirano, 1988) and in salmonids (Bolton *et al.*, 1986; Wagner and Mc Keown, 1986; Le Bail *et al.*, 1991). The sensitivity became a priority due to the low plasma GH levels found in the tilapia.

The hormonal specificity of the assay, analysed by examining cross-reactivity with other pituitary hormones, ensured that the antiserum did not react with any of the mammalian or teleostean PRL, GtH or TSH preparations. This greater hormonal specificity, not observed, at the same level, in previous tiGH RIAs (Farmer *et al.*, 1976; Ayson *et al.*, 1993), is probably due to the absence of pituitary hormone contamination in the rtiGH.

Brain and other tissue extracts from *O. niloticus* did not cross-react in rtiGH RIA, while plasma and pituitary extract of this genus gave inhibition slopes similar to the rtiGH standard. Thus native or recombinant GH can be measured without interference, in these different tissues, for concentrations as low as 0.1 ng/ml.

The zoological specificity has been studied on pituitary extracts from orders of fish which are more or less distant on the evolutionary scale. Our data showed that this assay is suitable for the measurement of immunoreactive GH in tilapia and probably all cichlid fish since the pituitary extracts from 9 cichlid species (data only shown for 5 species) showed parallel slopes of inhibition in the assay system. Pituitary dilutions from fish of the perciform order showed a partial immunological cross-reactivity. Pituitary extracts from forkbeard and sea scorpion, species belonging to orders quite close to the perciform one, exhibited a slight cross-reactivity, while fish from more distant orders showed only negligible or no interference in the assay system. Purified GH from seabream and trout showed curves similar to those exhibited by these species pituitary extracts. Thus, pituitary extract displacements were not artefactual. These data are in accordance with the phylogenetic distance.

The reliability of the rtiGH RIA is acceptable considering intra- and inter-assay variation coefficients. Tilapia GH RIA developed in the present study, using an antiserum against tilapia GH, was found to be both sensitive and highly specific, and suitable for the measurement of immunoreactive GH in plasma and pituitary of *O. niloticus* as well as in cichlids.

In order to evaluate the specific effect of water temperature on GH levels, for a short period, two experimental fish groups were held in 20 or 26°C water tanks under controlled conditions of photoperiod. The pituitary GH contents of the two groups, at the end of the experiment, were not significantly different but we observed a tendency to an increase of GH in warm water. These results agree with those reported by Yao (1993), in rainbow trout reared at three different temperatures: 8, 12 and 16°C. This author did not observe any difference between the pituitary GH contents of the different groups, although he noticed their fluctuations over time. However, the endogenic plasma GH levels increased significantly with the temperature throughout the experiment. It is obvious in our experiment that the decrease of the water temperature induced the decreases of plasma GH observed in colder-water acclimated tilapias. These GH level variations have been already reported by other authors, but on fish reared in conditions where both photoperiod and temperature varied (Marchant *et al.*, 1986; Barrett *et al.*, 1989; Björnsson *et al.*, 1989). Our results clearly support the hypothesis that GH could be involved, at least in part, in growth variations influenced by the water temperature. If it is the case, genetic selection linked to higher GH levels would allow the isolation of a strain more adapted to aquaculture in cold water.

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