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Environmental Control of Prolactin Synthesis in the Teleost Fish *Oreochromis* (Formerly *Sarotherodon*) *mossambicus*

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Accepted May 22, 1984

In the cichlid fish *Oreochromis mossambicus* prolactin cell activity is inversely related to the osmolarity and the Ca^{2+} concentration of the ambient water. Prolactin cell activity was estimated, at the end of a 3-week experimental period, by determination of the rate of prolactin synthesis during incubation of the rostral parts of the pituitary gland, in the presence of [^3H]lysine. Since the secretory activity of isolated prolactin cells is known to be inversely related to the osmolarity of the incubation medium, the possibility was investigated that the effects of changes in ionic composition of the ambient water on prolactin secretion *in vivo* are mediated by changes in osmolarity of the blood plasma. No support was found for this hypothesis. In fish exposed to high water osmolarities prolactin cell activity was reduced, while plasma osmolarity increased. In contrast, at high Ca^{2+} concentrations of the water, when prolactin secretion was inhibited to a similar extent, plasma osmolarity was significantly reduced. Although direct effects of plasma osmolarity on prolactin cells cannot be excluded completely, it is unlikely that plasma osmolarity is the predominant factor in the control of prolactin cell activity *in situ*. The physiological significance of the capacity of isolated prolactin cells to respond to changes in osmolarity of the ambient medium—a capacity shared with some other endocrine cell types—is therefore unclear. © 1985 Academic Press, Inc.

In general there is consensus about the osmolarity of the water as an important ambient factor controlling prolactin secretion in fish (Ensor and Ball, 1972; Dubourg *et al.*, 1980; Wendelaar Bonga and Van der Meij, 1981). For species such as sticklebacks and the cichlid *Oreochromis mossambicus* (further called tilapia), the water concentrations of Ca^{2+} and Mg^{2+} and the pH of the water are important additional factors (Wendelaar Bonga, 1978; Ogasawara and Yamada, 1979; Wendelaar Bonga and Van der Meij, 1980, 1981; Wendelaar Bonga *et al.*, 1983, 1984). However, species-specific differences have been reported for the responses of prolactin cells to changes in external Ca^{2+} levels (Dubourg *et al.*, 1983).

With respect to the internal control of prolactin secretion, two mechanisms have been proposed: neural control and regulation by blood factors acting directly on the prolactin cells, in particular plasma osmo-

larity. There is ample evidence for the presence of hypothalamic control (Wigham *et al.*, 1975; Peter and McKeown, 1975; Ball, 1981). The osmolarity of blood plasma could be a factor in mediating the effects of the osmolarity of the water on prolactin secretion (Sage, 1968; Nagahama *et al.*, 1975), since prolactin cells *in vitro* respond to osmolarity of the incubation medium (Sage, 1968; Nagahama *et al.*, 1975; Wigham *et al.*, 1977; Grau *et al.*, 1981). However it should be stressed that the evidence supporting this hypothesis is indirect and based on the study of prolactin cells that were deprived of their hypothalamic connections. The question remains to be answered whether prolactin cells *in situ* can respond autonomously to plasma osmolarity if the hypothalamic control mechanisms are intact.

In our studies on the control of prolactin secretion in tilapia we made several observations that were consistent with the con-

cept of direct control of prolactin cell activity by plasma osmolarity. But the results of some experiments raised doubt about this concept. If sticklebacks or tilapia were exposed to seawater with highly reduced concentrations of both Ca^{2+} and Mg^{2+} (but not in low- Ca^{2+} seawater; in this respect this work has often been misquoted), prolactin cells were activated even though plasma osmolarity increased (Wendelaar Bonga, 1978; Wendelaar Bonga and Van der Meij, 1980). In another experiment, fish were exposed to fresh water with high Ca^{2+} or Mg^{2+} levels. The resulting decrease in prolactin cell activity was accompanied by a drop instead of a rise in plasma osmolarity (Wendelaar Bonga and Van der Meij, 1980). In the experiments described in the present paper, plasma osmolarity was modified by exposing tilapia to fresh water with different osmolarities or different calcium concentrations. It was investigated whether there is a consistent relationship between plasma osmolarity and prolactin cell activity. While in previous experiments prolactin cell activity was estimated by morphometry at light and electron microscope levels, in this study prolactin cell activity was mainly assessed by determination of the rate of prolactin synthesis of the pituitary glands.

MATERIALS AND METHODS

Freshwater male tilapia (*O. mossambicus*; this name has recently been proposed for *Sarotherodon mossambicus*; Trewavas, 1981) of about 20 g were used. These fish were acclimated either to fresh water supplemented with NaCl or to artificial fresh water (for composition see Wendelaar Bonga and Van der Meij, 1980) with different Ca^{2+} concentrations. The osmolarity of the NaCl-enriched fresh water varied from 10, 165, 320, and 670 to 980 ($\pm 1\%$) mosmol/liter. The fish were adapted to these solutions in several steps, as described earlier (Wendelaar Bonga and Van der Meij, 1981). Transfer to water with different Ca^{2+} concentrations was performed in the following way: groups of fish were transferred from tapwater (Ca^{2+} , 0.8 mM) to artificial fresh water with the same Ca^{2+} concentration. After 2 days three groups of fish were transferred to water containing 0.2 mM Ca^{2+} , and 2 days later for two of these groups transfer followed to either 0.1 or

0.05 mM Ca^{2+} ; three other groups of fish were transferred directly to water with 2.5, 5.0, 7.5, or 10.2 mM Ca^{2+} ($\pm 2\%$).

After an exposure period of 21 days the fish were lightly anesthetized in MS-222. Blood was collected from the caudal blood vessels and the pituitary glands were dissected. Methods for determination of plasma osmolarity, for plasma total calcium, or for light microscopy have been described earlier (Wendelaar Bonga and Van der Meij, 1980). The glands were used either for light microscopy or for amino acid incorporation studies.

Pulse incubations. The rostral pars distalis was separated from freshly dissected pituitary glands and transferred to Dulbecco's modified Eagle's medium (MDM), as modified by Van Eys *et al.* (1983). The final osmolarity and Ca^{2+} concentrations were 310 mosmol/liter and 1.25 mM, respectively, similar to the osmolarity and free Ca concentration of the blood plasma of *S. mossambicus*. The prolactin lobes were placed in 100 μl MDM and preincubated for 1 hr 30 min in a metabolic shaker at 24°. Subsequently they were incubated in 100 μl MDM containing 15 μCi [^3H]lysine (New England Nuclear, sp act 90 Ci/mmol) for 3 hr at 24°. Afterwards the lobes were homogenized in 500 μl 0.1 M acetic acid in an all-glass homogenizer. The homogenate was centrifuged at 10,000g for 5 min in a Beckman microfuge and the supernatant was stored at -20° for later high-pressure liquid chromatography (HPLC) or freeze dried for later gel electrophoresis.

HPLC analysis. The 500- μl samples were analyzed with a Spectra Physics SP 8000 high-pressure liquid chromatograph (Spectra Physics, Eindhoven, The Netherlands) equipped with a stainless-steel column packed with Spherisorb 10 ODS (Chrompack BV, Middelburg, The Netherlands). The linear gradient consisted of a 0.5 M formic acid-0.14 M pyridine mixture (pH 3.0) and 1-propanol. The flow rate over the column was 2 ml/min, and 0.5-min fractions were collected with a LKB Redirac 2112 fraction collector. Four milliliters Aqua Luma (Baker Chemicals) was added and the fractions were counted in a Philips liquid scintillation analyzer (Model PW 4540).

Proteins were separated by sodium dodecyl sulfate (SDS)-gel electrophoresis after Laemmli, with 15% acrylamide, as described recently (Van Eys *et al.*, 1983). The gels were silver stained after Morissey (1981) and scanned with a Bio-Rad Model 1650 densitometer.

Statistics. The results were tested for statistical significance by Student's *t* test, at the 5% level.

RESULTS

Prolactin Synthesis in Vitro

After incubation of the rostral pars distalis of the pituitary glands (prolactin lobes)

in the presence of [^3H]lysine, homogenates of the lobes and the incubation media were analyzed by HPLC. Analysis of lobes of fish from normal fresh water (10 mosmol/liter; 0.8 mM Ca^{2+}) reveals only one major labeled and therefore newly synthesized product that elutes after 65 min. The same product is released in the incubation medium during incubation (Fig. 1). Only traces of this product were occasionally found after incubation of the proximal pars distalis, while it was absent after incubation of the pars intermedia. SDS electrophoresis of this peak resulted in two bands with M_r of 20.5K and 21.5K, which are typical for tilapia prolactin under our electrophoretic conditions (Wendelaar Bonga *et al.*, 1984). HPLC peaks eluting in the first 10 min are [^3H]lysine and small products representing contaminations of the [^3H]lysine preparation (Van Eys *et al.*, 1984). Ultrastructural examination showed that the prolactin lobes did not contain any growth hormone cells or other types of endocrine cells, except for some ACTH cells. Minute amounts of newly synthesized ACTH—eluting after

40 min under the chromatographic conditions used—could be observed (Fig. 1).

The total amount of labeled prolactin recovered after incubation from lobes and media reflects the hormone-synthesizing capacity of the prolactin lobes at the moment of dissection and is therefore used as a parameter for prolactin cell activity.

Effects of External Osmolarity and Ca^{2+} on Prolactin Synthesis

As shown in Fig. 2, exposure of fish for 3 weeks to fresh water enriched with NaCl leads to a marked reduction of prolactin cell activity. In a medium that is iso-osmotic with the blood plasma (330 mosmol/liter), the rate of prolactin synthesis is only 20% of that of fish from normal fresh water. At higher osmolarities the rate of prolactin synthesis is slightly but not significantly higher.

Exposure to water enriched with CaCl_2 induces a reduction that is even more pronounced if expressed on a molar basis. For a 50% reduction of prolactin synthesis, as obtained after exposure to 2.5 mM CaCl_2 (17 mosmol/liter), a concentration of 80 mM NaCl (165 mosmol/liter) is required (Fig. 2).

Of the fish exposed to the highest NaCl concentration (980 mosmol/liter) about 50% died in the course of the experiment. The fish of the other groups did not show enhanced mortality.

In both experimental series, prolactin cell size showed a similar decrease at higher osmolarities or Ca^{2+} concentrations as prolactin synthesis (Fig. 2).

Plasma Osmolarity and Total Ca

An increase in the NaCl concentration of the water leads to a small but consistent increase in plasma osmolarity (Fig. 3). The values for fish from water with an osmolarity of 980 mosmol/liter differed significantly from the control value ($P < 0.05$). Plasma total Ca did not change. Exposure of fish to high Ca^{2+} concentrations leads to a slight drop in plasma osmolarity, statisti-

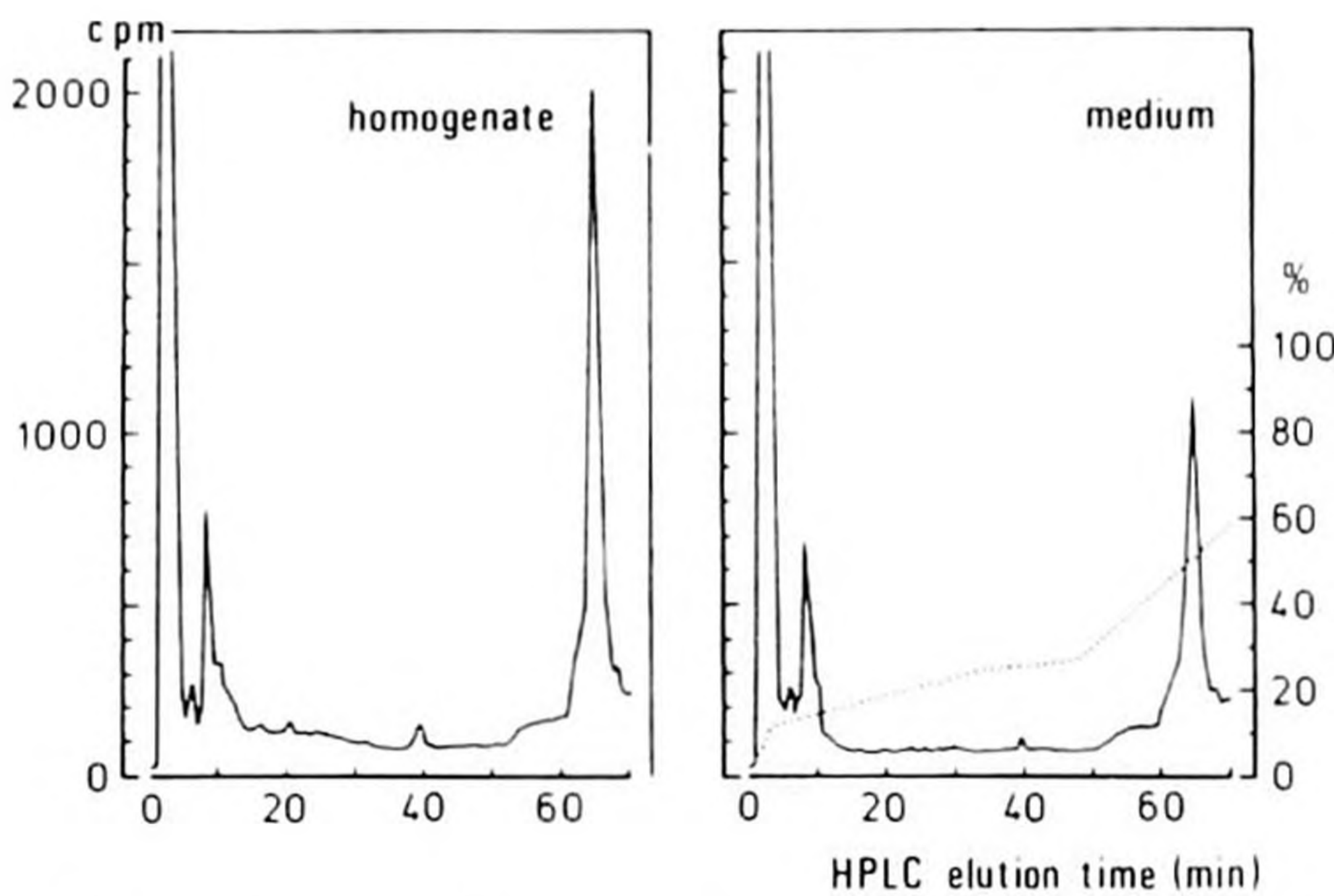


FIG. 1. High-performance liquid radiochromatogram of newly synthesized proteins in prolactin lobes (rostral pars distalis) of tilapia pituitary glands. Two lobes of freshwater tilapia were incubated for 3 hr in medium containing [^3H]lysine. Subsequently, extracts of the lobes (left) and the medium (right) were submitted to HPLC, and the radioactivity of the elution samples was determined. Peaks eluting within 10 min represent unincorporated [^3H]lysine and contamination; the peak eluting after 65 min was identified as prolactin by SDS-gel electrophoresis; dotted line is elution gradient as percentage of secondary solvent (1-propanol).

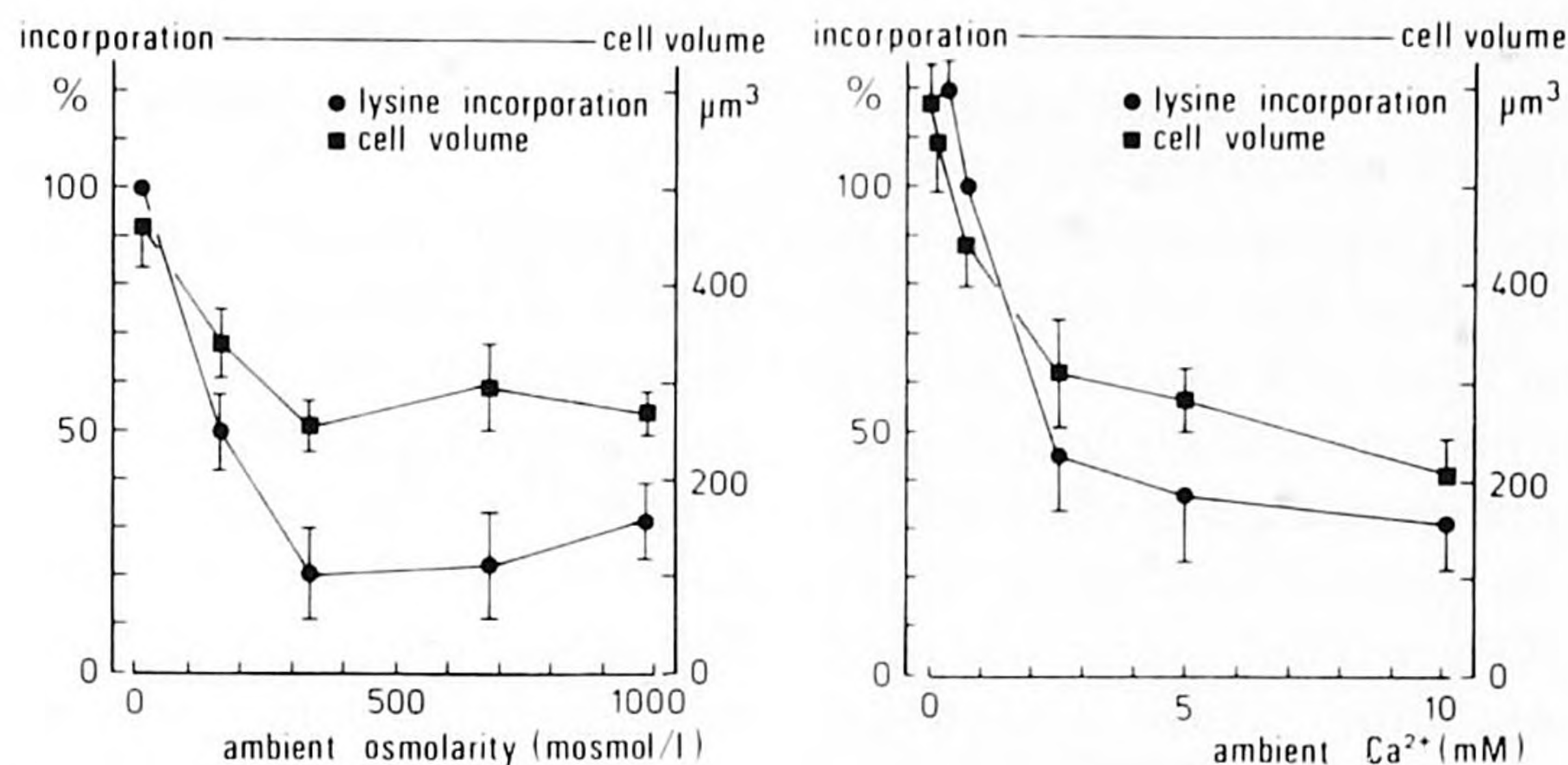


FIG. 2. Effect of water osmolarity (left; NaCl in fresh water) or Ca^{2+} concentration (right; CaCl_2 in fresh water) on prolactin cell volume and rate of prolactin synthesis. After exposure of the fish for 3 weeks the pituitary glands were removed and either processed for microscopy to determine prolactin cell volume (squares; $n = 6$) or incubated in the presence of [^3H]lysine; the amount of newly synthesized prolactin recovered from prolactin lobe homogenates and incubation media was estimated by determination of the radioactivity of the HPLC fractions eluting between 60 and 70 min (dots; $n = 4$; expressed as the percentage radioactivity found for fish from normal fresh water, 10 mosmol/liter; 0.8 mM Ca^{2+}).

cally significant at 5 mM Ca^{2+} and higher. If the Ca^{2+} concentration is reduced below normal freshwater levels, plasma osmolarity also decreases. Plasma total Ca levels show a complicated pattern. At ambient Ca^{2+} levels up from 7.5 mM it is significantly increased ($P < 0.05$), whereas at concentrations below the control level (0.8 mM Ca^{2+}) plasma Ca first increases and then drops rapidly (Fig. 3).

DISCUSSION

HPLC analysis of prolactin lobe homogenates and incubation media showed one major peak that yielded two peaks on SDS gel, with M_r values of 20.5K and 21.5K, that are typical for prolactin under our electrophoretic conditions and that appeared to be specific for the prolactin lobe and not for the other parts of the hypophysis. For tilapia prolactin slightly lower (Clarke, 1973; Farmer *et al.*, 1977) or higher (Specker *et al.*, 1984) values have been reported after SDS electrophoresis. We consider such differences typical for the SDS procedure, which involves denaturation and binding of an anionic detergent, treatments that may

affect electrophoretic mobility. We could modify the mobility of the 21.5-Da band to a value corresponding to 24 Da by prolonging the denaturation treatment from 1 (as usual) to 3 min. Apparently, SDS-gel electrophoresis is not the method of choice for determination of prolactin molecular weight in tilapia.

External factors controlling prolactin secretion. Osmolarity and Ca^{2+} concentration are probably important environmental factors in the control of prolactin secretion in freshwater tilapia. Increased levels of NaCl and CaCl_2 of the water lead to a marked decrease of prolactin cell activity. On a molar basis, CaCl_2 is much more effective than NaCl. Obviously, Ca and not Cl ions are the relevant factor, since there is no clear correlation between the chloride concentration and the rate of prolactin synthesis if the data on NaCl and CaCl_2 exposure are compared. Although we assume that the inhibitory effect of the NaCl-containing water on prolactin synthesis is caused by the osmolarity of the water, additional effects of either Na^+ or Cl^- cannot be ruled out completely.

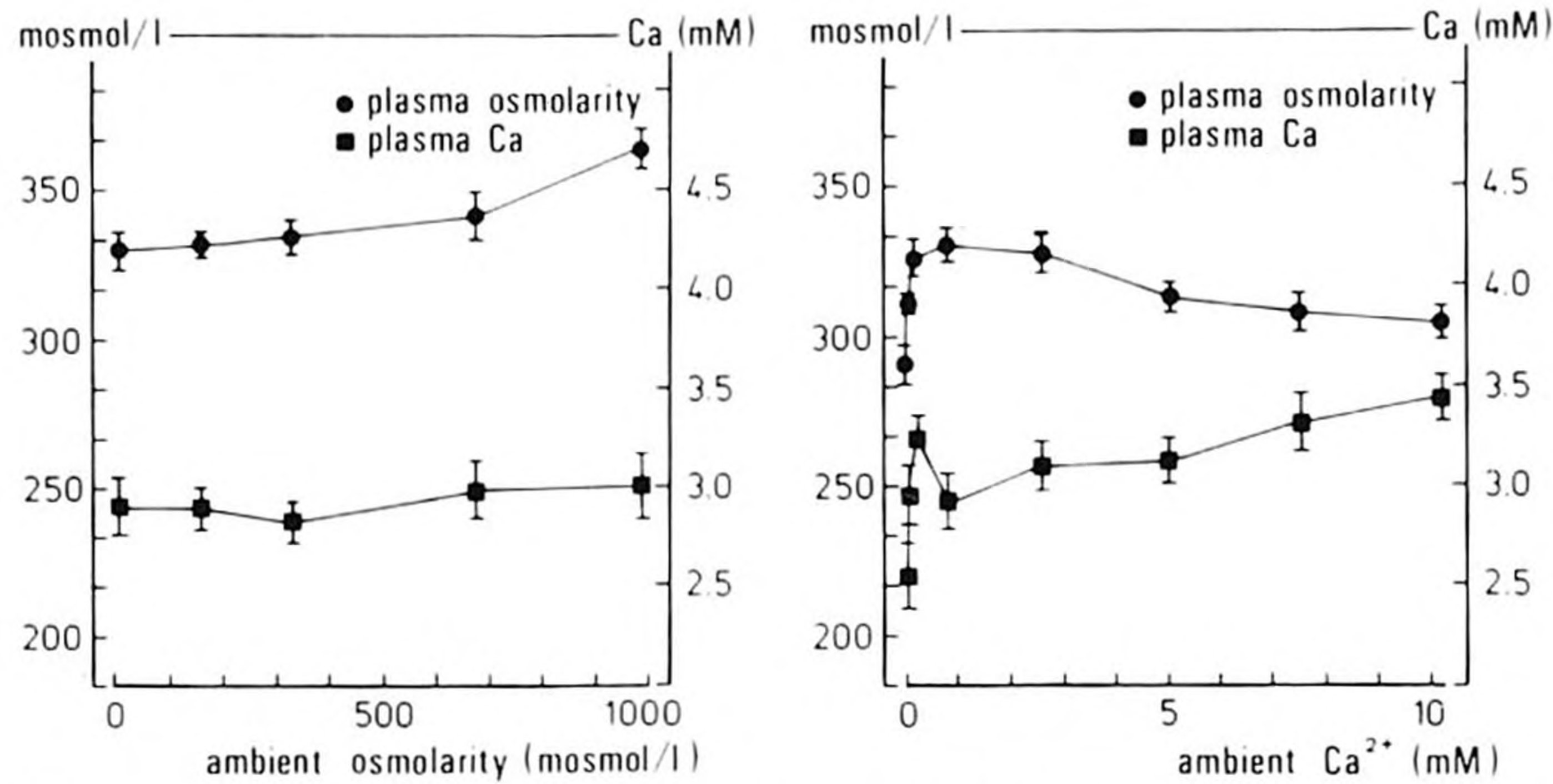


FIG. 3. Effect of water osmolarity (NaCl in fresh water) or ambient Ca^{2+} (CaCl_2 in fresh water) on plasma osmolarity (dots; $n = 8$) and plasma total Ca (squares; $n = 8$). Fish were acclimated for 3 weeks.

The marked reduction of prolactin synthesis shown by prolactin cells of fish exposed to high Ca^{2+} levels confirms earlier results obtained by ultrastructural morphometry (Wendelaar Bonga and Van der Meij, 1980, 1981). To a large extent the same conclusion can be drawn for the effects of ambient osmolarity. We have shown before that an increase in the osmolarity of fresh water leads to a reduction of prolactin cell activity as reflected by morphometrical analysis in tilapia (Wendelaar Bonga and Van der Meij, 1980, 1981). However, in these earlier experiments prolactin cells showed signs of high activity in fish adapted to water with an osmolarity higher than that of the blood plasma, if the Ca^{2+} and Mg^{2+} levels were kept low. This was not observed in the present study. The reason for this discrepancy may be connected with the strains of tilapia used.

Prolactin cell activity in tilapia *in vivo* is stimulated not only by low water osmolarity or Ca^{2+} levels, but also by low water pH (Wendelaar Bonga *et al.*, 1984). Reductions in water osmolarity, Ca^{2+} level, or pH have three effects in common: the branchial influx of water and the passive outflow of ions are initially increased, plasma osmolarity and ion concentrations are decreased, and prolactin cells become activated (Wen-

delaar Bonga *et al.*, 1984). Since prolactin reduces gill permeability to water and ions and increases plasma electrolyte levels in fish (Clarke and Bern, 1980), stimulation of prolactin secretion seems an adequate response to counteract the effects of reduced water osmolarity, Ca^{2+} concentrations, or pH.

Internal factors controlling prolactin secretion. If fish prolactin cells are incubated in culture media, their secretory activity is stimulated when the osmolarity of the incubation medium is reduced (Nagahama *et al.*, 1975; Wigham *et al.*, 1975, 1977; Benjamin and Baker, 1976, 1978; Grau *et al.*, 1981; our unpublished observations). This response has led to the suggestion that the stimulation of prolactin cells as observed after transfer of fish from seawater to fresh water is mediated by a reduction of plasma osmolarity. Our present data do not support this suggestion, since the reduction of plasma osmolarity in fish exposed to high water Ca^{2+} levels was accompanied by a reduction of prolactin synthesis. Thus it is unlikely that prolactin synthesis in tilapia is predominantly controlled by direct or indirect effects of plasma osmolarity on these cells. Brewer and McKeown (1980) came to the same conclusion for coho salmon, after they found that the stimulation of prolactin secretion following transfer of the fish from

seawater to fresh water was not accompanied by notable changes in plasma osmolarity.

Apparently, tilapia prolactin cells show a consistent response to osmolarity of the surrounding fluid only in the absence of intact hypothalamic control mechanisms. A similar conclusion can be drawn for prolactin cells of European eels and rats that are also stimulated *in vitro* after reduction of the osmolarity of the incubation medium (Benjamin and Baker, 1976, 1978; LaBella *et al.*, 1975). In contrast, if in European eels plasma osmolarity is reduced by exposing the fish to deionized water, the prolactin cells are unaffected (Oliverieu *et al.*, 1980, 1981). Similarly, plasma prolactin levels in rats are not noticeably influenced by experimentally induced increases or decreases in plasma osmolarity (Mattheij, 1977a,b). Thus, the physiological significance of the response of prolactin cells *in vitro* to changes in osmolarity seems unclear. This is the more so since such a response is not typical for prolactin cells. It has also been described for incubated ACTH and growth hormone cells of European eels and the molly *Poecilia latipinna* (Benjamin and Baker, 1978; Batten *et al.*, 1983) and bovine adrenal medullary cells (Hampton and Holz, 1983).

We have suggested that plasma calcium instead of plasma osmolarity may influence prolactin secretion in fish (Wendelaar Bonga and Greven, 1978). However, the present data show that there is no consistent relationship between plasma calcium levels and prolactin synthesis in tilapia. Although the presence of calcium ions may be a prerequisite for basal prolactin release (Grau *et al.*, 1981; MacDonald and McKeown, 1983), as it is for release in many gland cells, it is unlikely that the prolactin cells in tilapia are predominantly controlled by changes in plasma calcium levels.

In the present experiments a complicated relationship was found between the calcium concentrations of the water and blood

plasma. Although in general the plasma calcium levels are correlated positively to the external calcium concentration, a reduction in the calcium concentration of the water from 0.8 to 0.2 mM leads to an increase in plasma calcium. The most simple explanation is that plasma calcium levels are determined by both the calcium concentration of the water and the rate of secretion of a hypercalcemic hormone, for which prolactin is a likely candidate (Pang, 1981; Wendelaar Bonga and Flik, 1982).

We suggest that prolactin synthesis and release are mainly controlled by hypothalamic tracts, and not by direct effects of plasma factors on the prolactin cells. Whereas inhibitory control by dopaminergic fibers has been well established for teleost prolactin cells, there is growing evidence that there are in addition stimulatory tracts that may be serotonergic (Ball, 1981). The problem remains to be solved as to how information about the ionic composition of the water is mediated to the central nervous system. More attention should be paid to the sensory innervation of the branchial area, since Mayer-Gostan and Hirano (1976) showed that in eels transection of the vagal and glossopharyngeal nerves, which innervate the branchial area, has pronounced effects on osmoregulation. The authors suggested that efferent nerve fibers may send information from branchial receptors to the brain that may regulate the release of hormones.

ACKNOWLEDGMENTS

The authors are indebted to Mr. A. Coenen, Mr. G. H. Goyvaerts, and Mr. P. Crujisen for technical assistance.

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