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Source: Zoological Science, 14(2) : 353-357

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.353

ZOOLOGICAL SCIENCE **14**: 353–357 (1997)

Effect of Temperature and Photoperiod on Prolactin Transcription in *Cyprinus carpio*

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ABSTRACT—We studied the effect of temperature and photoperiod on prolactin (PRL) gene expression in the pituitaries of summer-aclimatized carp (*C. carpio*). To assess the level of PRL gene transcription, we used a 24mer synthetic oligonucleotide probe derived from the known carp PRL gene sequence. Employing *in situ* hybridization assays, we observed high expression of PRL mRNA in the rostral pars distalis of summer-acclimatized carp in contrast to the almost negligible PRL transcription which occurs in the winter-acclimatized fish. When experimental combinations of long and short photoperiods with 10°C and 20°C environmental temperatures were studied, only a short photoperiod (8L-16D) in summer-acclimatized carp acclimated to a winter temperature (10°C) markedly depressed PRL gene expression. Our observations indicate that photoperiod constitutes a particularly relevant modulator in the neuroendocrine cascade that activates PRL transcription in the carp.

INTRODUCTION

The basic mechanisms underlying the compensatory response by which eurythermal fish adjust for seasonal changes affecting their habitat, entails reprogramming of the gene expression process both at the transcriptional and translational level (Figueroa *et al.*, 1994; Goldspink, 1995; Krauskopf *et al.*, 1981; Plant *et al.*, 1977; Saez *et al.*, 1982). Carp (*Cyprinus carpio*) acclimatization involves seasonal modulation of ribosomal RNA transcription, which takes course in different tissues (Vera *et al.*, 1993). In addition, the quantitative expression of liver proteins such as apolipoprotein A-I (Inostroza *et al.*, 1990) and the estrogen induction of vitellogenesis (Hernández *et al.*, 1992) reach the lowest point in cold-acclimatized carp. Likewise, as reported by Goldspink (1995) in muscle of warm- relative to cold-acclimated carp, one myosin heavy chain isoform RNA increases. Because the adaptive response seems to require adjustments of the gene expression process in different tissues, we are working with the hypothesis that seasonal environmental transcription regulation occurs as a result of the transduction of signaling molecules whose synthesis is modulated in connection with the periodic external milieu changes.

Prolactin (PRL), the most versatile pituitary hormone (Sinha, 1995), has been associated with fresh water and salinity adaptation (Bern, 1983; Hirano, 1986; Yada *et al.*, 1992) and with confinement stress response in euryhaline teleosts (Pottinger *et al.*, 1992). It has also been well established that, in mammals, neuroendocrine mechanisms which sense the photoperiod lead to seasonal changes in PRL secretion (Curlewis, 1992). In order to assess if PRL expression was implicated in the acclimatization process, we studied the state of PRL gene expression during the seasonal adjustment of the carp using *in situ* hybridization assays, and we found high expression of PRL mRNA in the rostral pars distalis (RPD) of summer-acclimatized carp pituitary glands (Figueroa *et al.*, 1994). We observed negligible level of transcription in tissue sections of pituitary glands from the winter-acclimatized fish.

Because seasonal acclimatization of eurythermal fish encompasses molecular responses to changes pertaining to different environmental factors, particularly temperature and photoperiod, it is important to assess if both physical parameters concur in the conformation of the signal that triggers the reprogramming of gene expression. As PRL may be one of the signal molecules coordinating the biochemical adjustment in different tissues, in the present study, we examined, by means of *in situ* hybridization, the effect of temperature and photoperiod on PRL gene transcription in acclimated carp.

MATERIAL AND METHODS

Animals

Male carp (*Cyprinus carpio*) weighing 1,000-1,500 g were caught in the Calle-Calle River during summer and winter and kept in a fixed 3 × 4m cage submerged 2 m in an effluent of the same river. The temperature of the water in summer and winter was 18-20°C and 8-

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10°C respectively.

Acclimation of carp

Carp were acclimated in indoor fiber glass tanks (1.0 × 0.5 × 0.7 m) containing 250 l of river water under continuos oxygenation. Water was adjusted to 10°C or 20°C to mimic winter- or summer-environmental temperatures, respectively. Carp maintained in the river were transferred to the fiber glass tanks at the temperature corresponding to their natural environment and adjusted at 1°C per day. Lightbulbs (*Spotline*, Phillips) were used to emulate daylight. At water surface level a maximum of 5,000 lux was reached. The minimum light intensity at the most distant point was 1,000 lux. Long (16L-8D) and short (8L-16D) photoperiods were used to follow summer and winter conditions, respectively. The fish were kept for at least 21 days under each experimental condition.

Besides summer- and winter-acclimatized control carp, a total of 32 fish were used for this study in two trials. Groups of four carp were subjected to four acclimation conditions (10°C or 20°C combined with

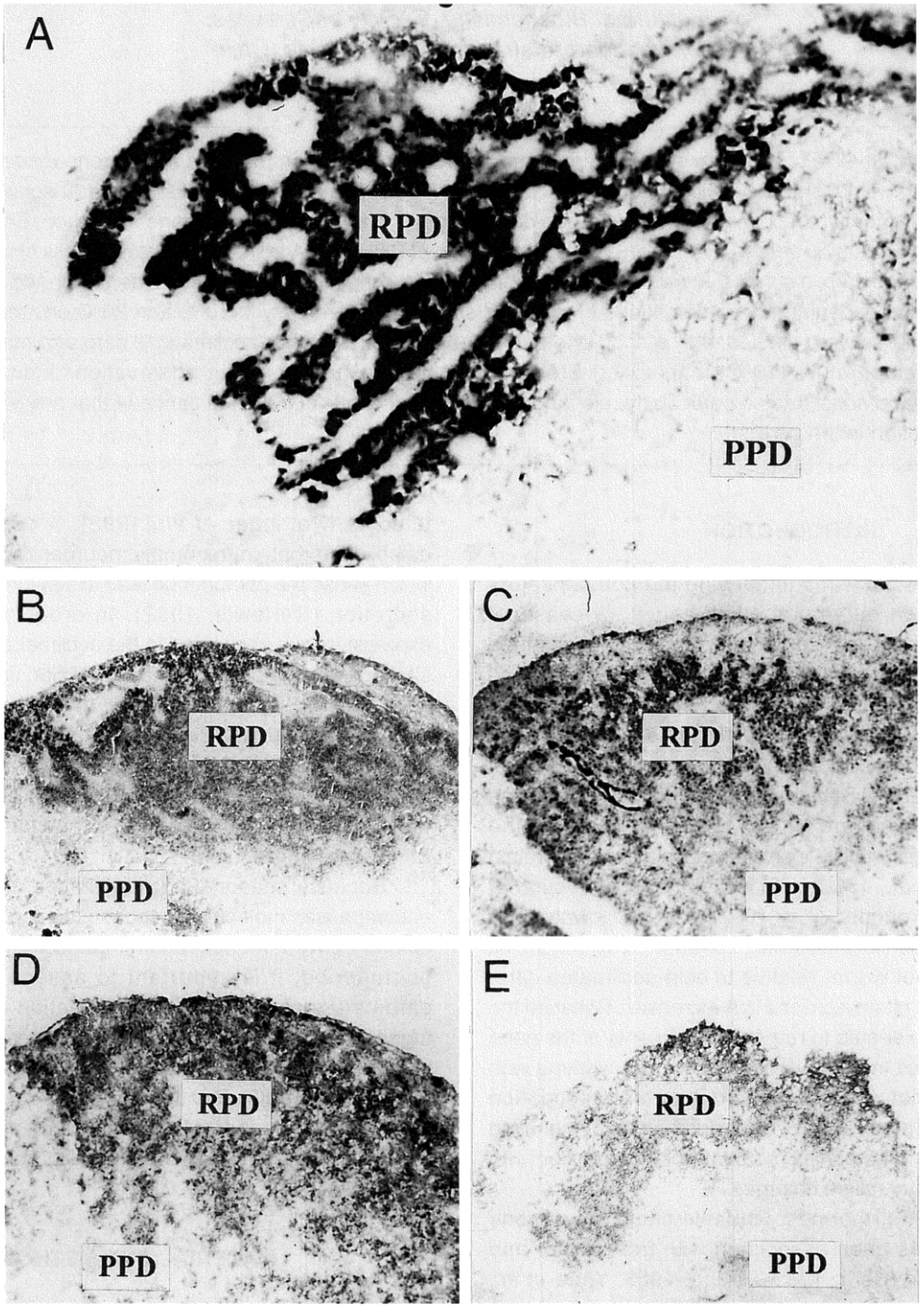

Fig. 1. *In situ* hybridization of sagital sections of carp pituitary glands from summer-acclimatized fish acclimated to cold and warm temperatures and long and short photoperiods. Molecular hybridization was obtained with a digoxigenin-labeled 24mer oligonucleotide antisense probe for carp PRL. (**A**) Control carp (× 100); acclimation to 20°C and (**B**) a photoperiod of 16L-8D (× 75) and (**C**) of 8L-16D (× 75); acclimation to 10°C and (**D**) a photoperiod of 16L-8D (× 75) and (**E**) of 8L-16D (× 75).

either 16L-8D or 8L-16D).

In situ *hybridization*

The pituitary glands from the control summer- and winter-acclimatized carp, as well as from each of the summer-carp subjected to the four different acclimation conditions, were quickly removed and immersed in 4% (w/v) paraformaldehyde in phosphate saline buffer pH 7.4 (PBS). Sections (12 μm) of the frozen fixed tissues were obtained and placed on slides pre-treated with a solution of 0.01% (w/v) poly-L-Lysine. Samples from each of the set of experiments covering all acclimation conditions were kept at -70°C and processed together. Immediately before hybridization with the oligonucleotide probes, the sections were postfixed with paraformaldehyde, washed in PBS and maintained for 10 min in 2 × standard saline citrate (SSC) (Figueroa *et al.*, 1994).

Synthetic 24mer oligonucleotides probes (sense and antisense), whose sequence included nucleotides G_{2041}-T_{2064} located in exon V of the carp PRL gene (Chen *et al.*, 1991; Figueroa *et al.*, 1994), were used. The sequences of the oligonucleotides were unique with respect to the carp growth hormone gene (Chiou *et al.*, 1990). The probes were labeled with digoxigenin-11-dUTP at the 3' end using terminal deoxynucleotidyl transferase and the *in situ* hybridization was carried out as described elsewhere (Figueroa *et al.*, 1994).

After hybridization, the slides were washed twice with 2 × SSC, once with 1 × SSC for 1 hr at room temperature, followed by a 30 min wash with 0.5 × SSC at 37°C and, lastly, by a 30 min rinse with 0.5 × SSC at room temperature. The immunological detection of the hybridized probes was accomplished as described (Figueroa *et al.*, 1994). Quantification of the label in the pituitary sections was carried out by a modification of the procedure described by Ayson *et al.*, (1994) using an automated image digitizing system (UN-SCAN-IT, Silk Scientific, Inc., USA). Six areas of approximately 0.2 mm^2 each were randomly selected in both the rostral pars distalis (RPD) and in the proximal pars distalis (PPD), the later yielding background values. In each of the 12 areas of each pituitary sagital section, the value of pixels representing the density of the label was quantified. The total pixel values representing each of the six areas examined in each microscopic image of the RPD were corrected by substracting the average value rendered by the pixels corresponding to the PPD areas. Statistical analyses were performed using the Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

The *in situ* hybridization signals for PRL mRNA were always localized in the RPD (Fig. 1). Molecular hybridization did not occur when the digoxigenine-labeled sense oligonucleotide probe derived from the carp PRL gene sequence was used.

As shown in Fig. 1A, high expression of PRL mRNA was observed in summer-acclimatized fish, whereas the winter-adapted carp exhibited a remarkably low hybridization (tissue section not shown, see Fig. 2F). The level of PRL mRNA in the pituitary glands from summer-acclimatized carp did not change upon acclimation at 20°C under long (16L-8D) and short (8L-16D) photoperiods (Fig. 1B, C). A long photoperiod likewise rendered comparable levels of PRL gene expression in 10°C acclimated fish (Fig. 1D). However, upon acclimation of the summer-acclimatized fish at 10°C, a short photoperiod produced a drastic decrease of PRL transcription (Fig. 1E). The quantification and statistical analysis of the level of PRL transcription attained in the summer-acclimatized carp and in each of the four acclimation conditions is shown in Fig. 2.

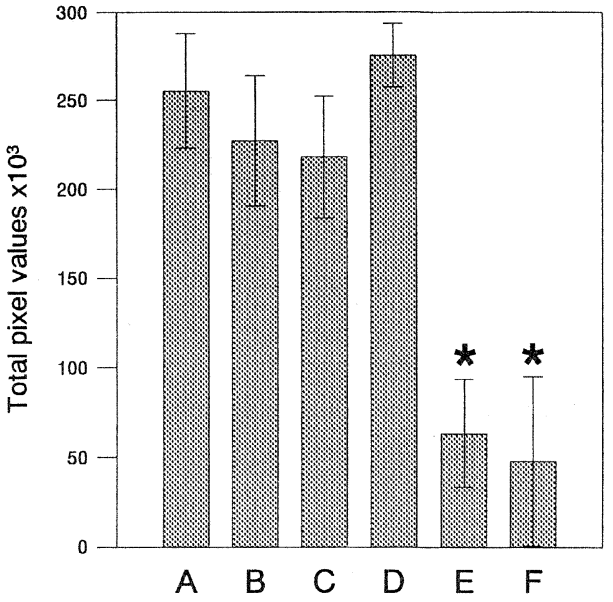

Fig. 2. Level of PRL gene expression of summer- and winter-acclimatized carp, and of summer-acclimatized fish acclimated to different temperatures and photoperiod. Each bar represents the mean ± standard deviation of the total pixel values measured in six different areas of the RPD of the carp pituitary sections. (**A**) Control: Summer-acclimatized carp; (**B**) acclimation to 20°C and to a photoperiod of 16L-8D, (**C**) acclimation to 20°C and to a photoperiod of 8L-16D, (**D**) acclimation to 10°C with a photoperiod of 16L-8D, (**E**) acclimation to 10°C with a photoperiod of 8L-16D; (**F**) Winter-acclimatized carp. Asterisks indicate a significant difference with the summer-acclimatized carp (**A**) (*t*-test. $P < 0.05$; $n = 4$).

Also, the level of the *in situ* hybridization signals for PRL mRNA in pituitaries from winter-acclimatized carp (Fig. 2F). Clearly, acclimation of summer-acclimatized fish to cold temperature and a short photoperiod (Fig. 2E) renders a significant difference in PRL transcription, comparable to that attained during the natural seasonal condition of the fish (Fig. 2F).

DISCUSSION

Both acclimation and acclimatization studies have, in general, favored the consideration of temperature as the direct effector of compensatory responses in fish. Concerning shifts in gene expression, it is clear that reprogramming may be brought about by metabolic effects, by differences in the hormone levels, photoperiod, and even by mechanical activity (Figueroa *et al.*, 1994; Goldspink, 1995).

In euryhaline teleosts, PRL plays a substantial role in maintaining hydromineral balance in freshwater (Bern, 1983; Brown and Brown, 1987; Hirano, 1986; Hirano *et al.*, 1987). Utilizing *in situ* hybridization, Nishioka *et al.* (1993) found that either tilapia PRL_{177} or tilapia PRL_{188} gene expression varied according to the environmental salinity from which the tilapia (*Oreochromis mossambicus*) pituitaries were removed. *In situ* hybridization signal was significantly stronger in freshwater pituitaries than in seawater pituitaries.

In the present study, acclimation of carp, although known to be insufficient to mimic the complexity of acclimatization (Segner and Braunbeck, 1990), provided an unique opportunity to ascertain the effects of temperature and photoperiod on PRL gene transcription. All the acclimation experiments were carried out with male carp to elude hormonal changes linked with the reproductive cycle that takes place throughout seasonal changes.

Using *in situ* hybridization, we were able to detect a profound photoperiodic effect in summer-acclimatized carp acclimated to the winter temperature. While changes in the photoperiod did not significantly affect summer fish acclimated to their season's temperature, a short photoperiod (8L-16D) remarkably depressed PRL transcription in the rostral pars distalis of the carp acclimated to the winter temperature (Fig. 1E). Acclimation of the summer-acclimatized carp to winter conditions, i.e. low temperature and a short photoperiod, resulted in a PRL gene expression level comparable to the physiological manifestation attained during the cold season (Fig. 2F). While photoperiod by itself at the corresponding summer temperature is insufficient to determine changes in PRL transcription, it certainly plays an important role in the acclimation of summer-acclimatized carp to winter temperatures.

Earlier acclimation studies by McKeown and Peter (1976) showed that in goldfish, *Carassius auratus*, longer photoperiods and higher temperatures caused pituitary PRL release. Serum PRL changed on a circadian rhythm depending on the length of the photoperiod (McKeown and Peter, 1976). In other fish, such as the trout *Onchorhynchus mykiss*, plasma PRL levels do not show a pronounced annual cycle (Rand-Weaver *et al.*, 1995). However, in male and female trout, PRL levels were inversely correlated to water temperature (Rand-Weaver *et al.*, 1995).

It is well known that all circadian systems imply a certain degree of cell autonomy and that a photic entrainment pathway is always present as an input (Takahashi, 1993). It is also well established that seasonally breeding eutherian species show the characteristic pattern of high plasma PRL level in spring and summer and a low concentration in the colder seasons, with photoperiod being the major environmental factor controlling this circannual pattern (Curlewis, 1992). Although it is known that in mammals the photoperiod acting via pineal melatonin secretion is involved in the control of the seasonal changes in plasma PRL levels, the mechanism by which melatonin interacts with the lactotrophs remains unclear. In carp, plasma melatonin levels have been assessed for daily cycles and the observed rhythm suggests that melatonin is an important hormone in photoperiodism and circadian rhythm in the fish (Kezuka *et al.*, 1988). Our observations in acclimated carp have been focused on the first measurable event of PRL gene expression and suggest that the photic stimuli constitute a particularly relevant modulator in the neuroendocrine cascade that activates PRL transcription.

ACKNOWLEDGMENTS

We would like to thank Ms. Susan Watson and Prof. Oriana Gonzalez for their critical reading of the manuscript. This work was supported by grants 1940845 from FONDECYT and GE/GLO/90/004 UNIDO/ICGEB.

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(Received March 13, 1996 / Accepted November 20, 1996)