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Environmental Acclimatization of the Carp Modulates the Transcription of β-Actin

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Abstract A cascade of mechanisms involving changes in gene expression are substantial to shape the adaptive responses that a eurythermal fish requires upon environmental changes in its habitat. We have previously shown that the cyclical reprogramming of rRNA transcription is a remarkable feature in carp under seasonal acclimatization. Using in situ hybridization and competitive RT-PCR we found significant differences in β-actin transcripts, generally accepted to be coded by a typical housekeeping gene, in tissues from summer- and winter-acclimatized carp. The physiological differential β-actin transcription herein reported places us on the alert for the reference genes estimated to be constitutive to quantitatively assess gene transcripts. J. Cell. Biochem. 80:223–228, 2000. © 2000 Wiley-Liss, Inc.

Key words: β-actin transcription; gene expression quantification; housekeeping genes; carp; fish; acclimatization

Seasonal acclimatization, a process that allows eurythermal fish to compensate for the naturally occurring changes in environmental temperatures and photoperiod, is genetically programmed and involves a cyclical rearrangement of molecular and cellular functions and a regulation of transcriptional and translational events [Oñate et al., 1987; Vera et al., 1993; Figueroa et al., 1994; Vera et al., 1997; Kausel et al., 1999a]. Acclimation, although a distinctive process [Segner and Braunbeck, 1990], also requires molecular and gene expression adjustments to provide the homeostatic condition that fish need to survive [Segner and Braunbeck, 1990; Goldspink, 1995; Tiku et al., 1996; Hirayama and Watabe, 1997; Figueroa et al., 1997; Hirayama et al., 1998; Arends et al., 1998]. Current research on the complex physiological adaptive mechanisms underlying fish acclimatization and acclimation includes: 1) studies on the neuroendocrine cascade triggered by changes in ambient temperature, which at the same time entails transcriptional modulation in the hypothalamic-pituitary axis [Figueroa et al., 1997, 1999; Arends et al., 1998; Kausel et al., 1999]; 2) assessment of muscle [Goldspink, 1995; Hirayama and Watabe, 1997] and cell membrane fluidity responses [Tiku et al., 1996] associated with the regulation of the expression of specific genes; and 3) the characterization of the molecular events that sustain the generalized nucleolar rearrangement which reveal profound changes in ribosomal biogenesis as a consequence of the seasonal environmental variations [Vera et al., 1993, 1997; Kausel et al., 1999].

The products of housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase, β-actin, and 18 S rRNA have been widely used as a reference for specific mRNA quantification [Serazin-Leroy et al., 1998]. We recently reported that in the carp, and consistent with the seasonal nucleolar reprogramming [Vera et al., 1993], 5.8 rRNA is differentially expressed between summer and winter and that rRNA transcription should be used with caution as a reference to quantitate transcripts *vis à vis* the acclimatization process [Vera et al., 1997]. Here we present evidence that in situ hybridization of β-actin mRNA in different tissues from seasonal-acclimatized carp, as well as quantitative RT-PCR analyses, reveals substantial changes in the β-actin transcript levels between summer and winter. This indicates

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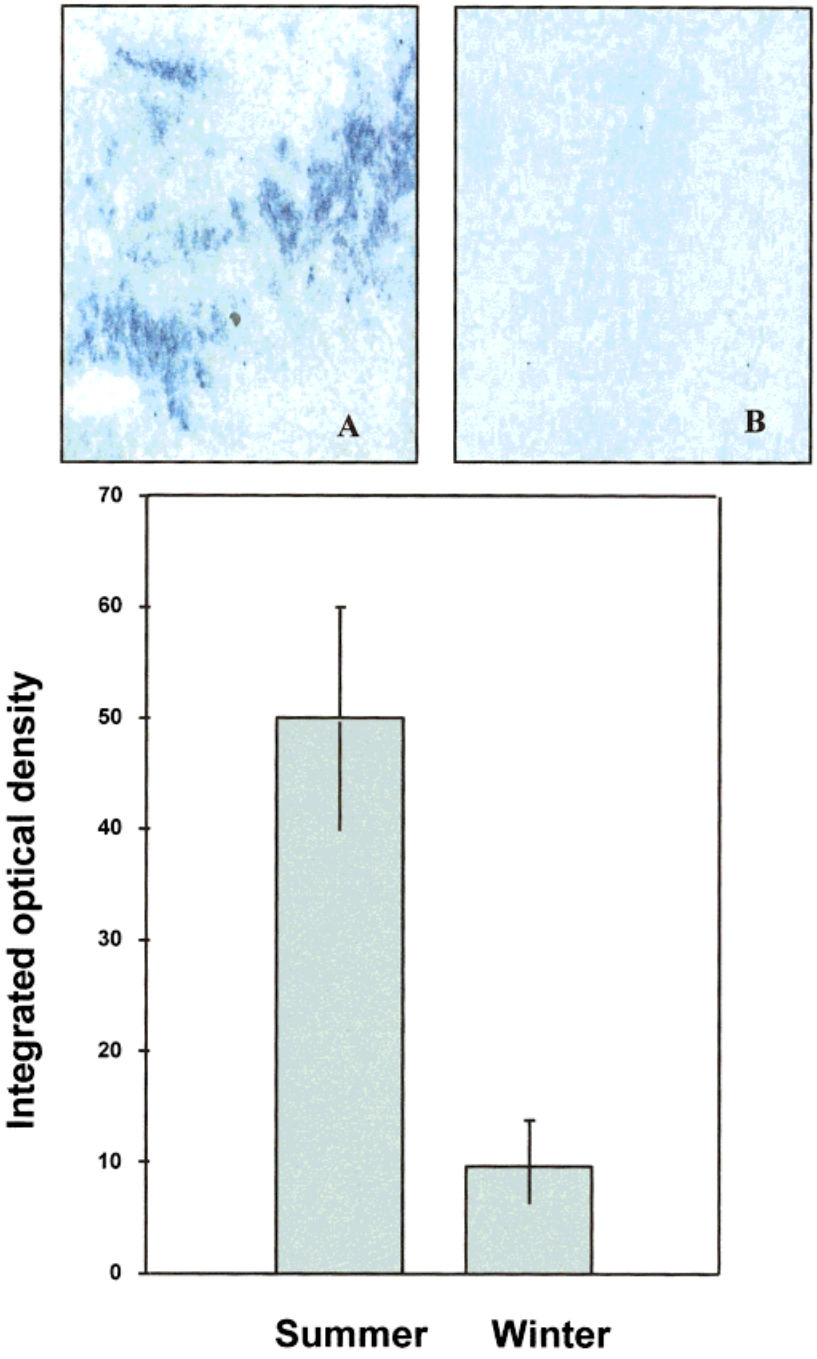

Fig. 1. In situ hybridization of brain sections of (**A**) summer- and (**B**) winter-acclimatized carp (×75). Molecular hybridization was attained with a digoxigenin-labeled 20mer antisense oligonucleotide specific carp β-actine (β*act3305an*) probe. The histogram depicts the mean integrated optical density of the in situ hybridization signals (n = 3/season). Student's *t*-test $P < 0.05$.

that the modulation of β-actin expression is involved in the cellular adaptive process and that β-actin cannot be considered a constitutive gene in the carp.

MATERIALS AND METHODS

Male carp (*Cyprinus carpio*) weighing 1,000–1,500 g were caught during winter and summer and maintained in a fixed 3 × 4 m cage submerged 2 m in an affluent of the same river with temperatures of 18–20°C (summer) and 8–10°C (winter). Brain, liver, and kidney, from summer- and winter-acclimatized carp were dissected and either frozen in liquid nitrogen and stored at −70°C for RNA extraction [Chomczynski and Sacchi, 1987] or fixed immediately for in situ hybridization studies, as reported elsewhere [Vera et al., 1997].

TABLE I. Quantification of β-Actin mRNA by In Situ Hybridization*

	Integrated optical density		β-actin mRNA
	Summer	Winter	Summer/Winter
Brain	50.06	9.65	5.20
Liver	58.34	19.34	3.02
Kidney	233.05	103.89	2.24

*β-actin mRNA level was calculated measuring the integrated optical density of the in situ hybridization signals in tissue sections obtained from four carp/season. Significant differences were attained between summer- and winter-acclimatized carp. Student's *t*-test $P < 0.05$.

Cloning of Carp β-Actin cDNA

A clone containing the full length of β-actin cDNA was obtained by RT-PCR, using as primers the oligonucleotides derived from the known carp β-actin gene sequence [Liu et al., 1990], 5′-CCTCATTTGAGCTCCT-3′ (sense) and 5′-GGATGTCTTACATGTGCA-3′ (antisense), and carp brain RNA (0.5 μg) as the template. The conditions for reverse transcription with Superscript II RT (Gibco-BRL, St. Louis, MO) and the 30 cycles of amplification with Taq DNA polymerase (93°C for 1 min, 55°C for 1 min, and 72°C for 90 s) were essentially those described by Kausel et al. [1999]. The reaction yielded a single amplification product of aproximately 1,800 bp, which was ligated to the TA-cloning vector pCR2.1 (Invitrogen, La Jolla, CA). The recombinant pβA24 contained the whole carp β-actin cDNA, which was confirmed by restriction mapping. Northern analyses, and corroboration of the expected size of the PCR amplification products using four different pairs of primers derived from the carp β-actin gene sequence [Liu et al., 1990].

Competitive RT-PCR

For the quantitative RT-PCR reactions [Siebert and Larrick, 1993], the sense (β*act2942se*); 5′-GGACCTGTATGCCAACACTG-3′) and antisense (β*act3305an*) (5′-GTCGGC-GTGAAGTGGTAACA-3′) oligonucleotides complementary to exons V and VI of the carp β-actin gene sequence [Liu et al., 1990] were used as primers. When reverse transcribed carp RNA was the template, a product of 282 bp was attained, while genomic DNA yielded a fragment of 383 bp containing the sequence of intron V. A competitive β-actin DNA was constructed as reported by Kausel et al. [1999], except that the 412 bp *Hind*III-*Eco*RI-fragment of a carp ribosomal RNA clone [Vera et al., 1997] was ligated to the oligonucleotides β*act2942se* and β*act3305an* and the phosphorylated complementary oligonucleotides 5′-AGCTCAGTGTTGGCATACAGGTCC3-3′ and 5′-AATTTGTTACCACTTCACGCCGAC-3′ harboring *Hind*III- or *Eco*RI-compatible ends, respectively. The resulting competitive β-actin DNA was further amplified using β*act2942se* and β*act3305an* as primers, and dilutions were prepared in 50 μg/ml glycogen.

Aliquots containing one-tenth of the total RNA (5 μg) isolated from the brain, liver, and kidney of summer- and winter-acclimatized carp, digested with 2U of Dnase I free Rnase, were reverse transcribed with Superscript II and oligo dT_{15}, and the competitive PCR assays were carried out using the β*act2942se* and β*act3305an* primers with 1/100 of the cDNAs obtained plus a serial dilution of the competitive β-actin DNA as templates. The amplification products were fractionated on agarose gels and quantified with an analytical Imager System (UN-SCAN-IT).

In Situ Hybridization

Sections (12 μm) of the frozen fixed tissues from the summer- and winter-acclimatized carp were processed simultaneously as described previously [Figueroa et al., 1997]. Molecular hybridization was carried out using the 3′-end digoxigenin labeled [Kaussel et al., 1999] 20mer oligonucleotide β*act3305an* and as a control, the equally-labeled 20mer sense oligonucleotide β*act2942se*. Quantification of the label in the tissue sections was obtained with an automated image digitizing system (IMAGE PRO PLUS, Media Cybernatics). The Student's *t*-test was used to assess differences. $P < 0.05$.

RESULTS AND DISCUSSION

To assess the level of β-actin transcription during acclimatization, in situ hybridization of brain sections was performed because in the carp this tissue contains the highest amount of the corresponding mRNA [Liu et al., 1990] (Fig. 1). The brain sections obtained from the summer-acclimatized fish clearly exhibited remarkable differences when compared to those

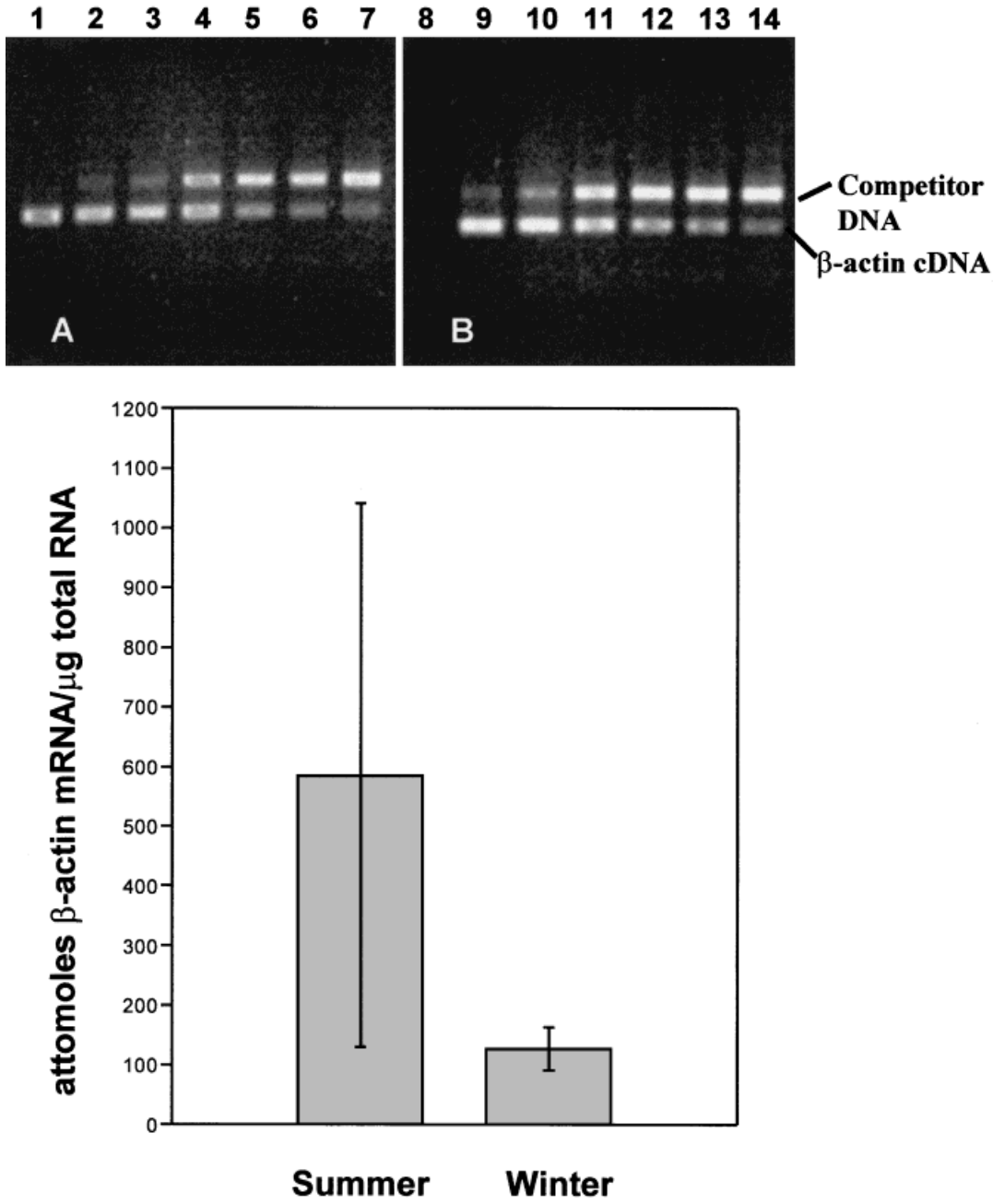

Fig. 2. RT-PCR of β-actin transcripts in brain from summer- and winter-acclimatized carp. Amplification products of competitive PCR obtained with the carp β-actin specific primers β*act2942se* and β*act3305an* with a constant amount of cDNA reverse transcribed from (**A**) summer- and (**B**) winter-acclimatized fish, complemented with dilutions of competitor DNA. Fractionation on a 1.4% agarose gel. *Lane 1* contains a sample of the constant amount of cDNA used in the assay and *lane 8,* no template. *Lanes 2* and *9, 3* and *10, 4* and *11, 5* and *12, 6* and *13,* and *7* and *14,* 0.05, 0.1, 0.5, 1.0, 2.0, and 4.0 attomoles of competitor DNA, respectively. The histogram depicts the amount of transcripts detected after analyzing eight carp brains for each acclimatized season. Student's *t*-test $P < 0.05$. In situ hybridization of brain sections of (A) summer- and (B) winter-acclimatized carp. Molecular hybridization was attained with a digoxigenin-labeled 20mer antisense oligonucleotide specific carp β-actine (β*act3305an*) probe. The histogram depicts the mean integrated optical density of the in situ hybridization signals (n = 3/season). Student's *t*-test $P < 0.05$.

TABLE II. Quantification of β-Actin mRNA by Competitive RT-PCR*

	β-actin cDNA (attomoles/μg total RNA)		β-actin cDNA
	Summer	Winter	Summer/Winter
Brain	935.87 (8)	203.84 (8)	4.59
Liver	169.41 (8)	47.81 (8)	3.54
Kidney	1310.20 (9)	439.04 (9)	2.98
Muscle	313.60 (9)	102.40 (9)	3.06

*The mean β-actin cDNA values were obtained from the number of individuals indicated in brackets. Significant differences were attained between summer- and winter-acclimatized carp. Student's *t*-test $P < 0.05$.

from the winter-adapted carp, where the hybridization signals were negligible. The specificity of the molecular hybridization assay was confirmed using the sense probe β*act2942se,* which did not render any signal. A summer-acclimatized/winter-acclimatized carp ratio of 5.2 was attained upon quantification of the labeled β-actin transcripts (Fig. 1, Table I). Seasonal changes in the expression of β-actin also occurred in other tissues as depicted in Table I.

To confirm the results obtained by in situ hybridization, the β-actin mRNA content in the brain, liver, kidney, and muscle was determined in seasonal-acclimatized fish by competitive RT-PCR. This quantitative approach confirmed that the expression of β-actin is strongly affected by the acclimatization process, which was higher during summer in all the tissues studied (Fig. 2, Table II) and rendered a summer to winter β-actin mRNA ratio of 4.59 for brain. The differences observed in all the tissues examined by the two quantitative approaches indicate that although widely considered to be a constitutive gene, the transcription of β-actin is clearly modulated by the process of seasonal acclimatization. In vitro studies have shown that osmolarity in rat liver cells [Husson et al., 1996], mitogens in thyrocites [Savonet et al., 1997], insulin in hepatoma cells [Messina, 1994], and 3T3-L1 cells differentiating into adipocytes [Cornelius et al., 1988] affect β-actin expression and that actin synthesis is autorregulated by transcriptional and post-transcriptional mechanisms [Reuner et al., 1995]. To our knowledge, the modulation of β-actin transcription in seasonally acclimatized carp represents the first evidence that a physiological process imposes remarkable changes in the expression of this gene, which is generally accepted as a typical housekeeping gene. Thus, the use of in situ hybridization and competitive RT-PCR [Siebert and Larrick, 1993], which avoids normalization in reference to genes estimated to be constitutive, seems to be clearly advantageous in quantitatively assessing gene transcripts.

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