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Neuroendocrine modulation of stress response in the anuran, *Rana esculenta*

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Abstract. In amphibians, as in other vertebrates, stress stimuli have been found to affect different functions, including development, growth, and reproduction. A wide range of responsiveness to stressors has been reported for amphibians; for instance, capture and/or captivity stress induced changes both in the hypothalamus-pituitary-interrenal and hypothalamus-pituitary-gonadal axes. However, few studies have examined the response to stress in terms of recovery and/or adaptation by applying stress paradigms for a short and long-term duration. In the present paper, the short-term captivity stress responses were evaluated in the anuran *Rana esculenta* by measuring peripheral corticosterone, androgens, prolactin (PRL), and growth hormone (GH) changes. Moreover, in long-term captivity and salinity stress, effects were evaluated by measuring peripheral PRL changes and those of PRL mRNA in the pituitary together with plasma corticosterone and androgens. Short-term (24 h) captivity stress induced an increase of peripheral corticosterone together with that of GH and PRL since these hormones are involved in the "alarm phase" and in energy demand of stressed animals. The opposite trend was found for peripheral androgens, in view of the negative effects exerted by stress in the reproductive axis. In long-term (1 month) captivity and salinity stress, responses were consistent with the increasing of PRL mRNA at pituitary level, through a long-loop feedback mechanism depending on the decreasing levels of peripheral PRL, whereas no changes were found in the levels of plasma corticosterone and androgens. Therefore, it seems that *Rana esculenta* activates different neuroendocrine mechanisms depending on the duration of stress and on the types of stressors.

Introduction

In every vertebrate class, the major stress-related hormones are produced in, or released from, chromaffin tissue, the interrenal gland (adrenal cortex), and the pituitary gland. In all cases, the secretion of these hormones increases in response to stressors (Greenburg and Wingfield, 1987). Acute stressors elicit an alarm reaction (flight or fight) by means of hypothalamic activation of the sympathetic nervous system; chromaffin activation releases the catecholamines. Adrenal corticotropin hormone (ACTH) is derived from proopiomelanocortin (POMC) and synthesized in the pars distalis and in the melanotropes of the pars intermedia. In response to the hypothalamic polypeptide, corticotropin-releasing-factor (CRF), ACTH is released from the pars distalis and stimulates the release of interrenal corticosteroids (most notably corticosterone in amphibians). This response is associated with the general adaptation syndrome in which a rapid initial "alarm" is followed by sustained glucocorticoid secretion. Moreover, endogenous POMC-derived peptides, such as β-endorphin (β-EP) and melanotropin (α-MSH), have been found to play an important role in behavioral and physiological responses to stressors (Mosconi et al., 1994a, 1998).

In amphibians, as in fish (Pottinger and Carrick, 1999; Kubokawa et al., 1999, Balm et al., 1994; Sumpter et al., 1985; Mosconi et al., 1994a, 1994b), stress stimuli have been found to affect different functions, including development, growth, and reproduction. Capture and/or captivity stress induced changes both in the hypothalamus-pituitary-interrenal and in the hypothalamus-pituitary-gonadal axes (Licht et al., 1983; Mendoça et al., 1985; Masanori and Ishii, 1990; Paolucci et al., 1990; Zerani et al., 1991; Moore and Miller, 1984; Mosconi et al.,

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1994a). In addition, a wide range of stressors has been reported for amphibians. For example, shaking and handling stress induced a significant increase in CRF-ir in parvocellular neurons of the anterior preoptic area of *Xenopus laevis* (Yao et al., 2004). Moreover, the role of corticoids in mediating the response of *Rana pipiens* tadpoles to intraspecific competition has been documented (Glennemeier and Denver, 2002), while interrenal stress response exhibited by amphibians following exposure to coal combustion waste (Hopkins et al., 1997) and xenobiotics (Hopkins et al., 1999) has also been reported. The latter aspect should be taken into account since the activation of the stress neuroendocrine axis in response to ecotoxicological impairment may be related to the dramatic decrease of wild amphibian populations around the world, because neuroendocrine disturbances decrease the ability of the immune system to protect animals against several types of diseases (Christin et al., 2003). Furthermore, increased secretion of corticosteroids has been associated with inhibited reproductive function not only in amphibians (Coddington and Cree, 1995; Mosconi et al., 1994a) but also in other vertebrate species (Carnevali and Mosconi, 1999; Lance et al., 2004). In addition, an elevation of plasma corticosteroids was found in fish closely related with an elevation of GH levels (Pickering et al., 1991). Moreover, it has also been found that stress responses involve the activation of the osmoregulatory system and an elevation of plasma prolactin, since amphibians spend a long period of their life in the water (Mosconi et al., 2000). Nevertheless, few studies have examined the response of amphibians to stress in terms of recovery and/or adaptation by applying stress paradigms for a short and long-term duration.

The present paper aims to give more insights into these issues, by applying two types of stress paradigms in the anuran amphibian, *Rana esculenta*. For the short-term paradigm, the stress responses were evaluated after 10 min, 30 min, 1 h, 10 h, and 24 h of captivity by measuring peripheral corticosterone. Androgens, prolactin (PRL), and growth hormone (GH) changes were also measured to determine how short-term stress affects endocrine functions associated with reproduction, osmoregulation and growth. In long-term captivity and salinity stress, effects were evaluated by measuring plasma PRL changes and those of PRL mRNA in the pituitary after 1 week and 1 month; plasma corticosterone and androgens were also evaluated.

Materials and methods

Animals

Male *Rana esculenta* frogs (body weight 23-25 g) were collected in June between 15:00 and 17:00 h at Colfiorito (Umbria, Italy, 820 m a.s.l.; 43°03′N, 12°55′E). The wild population of this anuran living in the mountain pond with fresh water (at 820 m a.s.l.) shows a well-defined reproductive cycle, in which the environmental cues play a pivotal role. The breeding period occurs in late spring (May), then a refractory period intervenes during the summer months followed by the autumn recovery and winter rest (Polzonetti-Magni et al., 1997). This site is characterized by agricultural land use, mainly potato growing; the predators for frogs are represented (A. Brusaferro, pers. comm.) by mammals (*Vulpes vulpes*) and ichthyophagous birds (*Botarus stellaris, Hyxobrynchus minutus, Ardea purpurea, Ardea Cinerea*). Two experimental designs were used to test the effects of short-term and long-term stress. In the short-term stress paradigms 30 animals were captured: 5 animals were immediately sampled as control, the other were divided over 5 different tanks (stocking density 1 animal/0.1 m^2) and sampled after 10 min, 30 min, 1 h, 10 h, and 24 h.

In the long-term stress paradigms 25 animals were captured and taken to the laboratory. Five animals were sampled as control, the other were divided over 4 different tanks at the same density as that described above. Two experimental groups were maintained in fresh water, the others in saltwater (2‰ of salinity).

We sampled blood from these animals 1 week and 1 month after capture. After general anesthesia with 0.05% solution of 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222; Sigma Chemical Company, St. Louis, MO), blood samples were taken using a heparinized syringe by cardiac puncture. Blood samples were centrifuged at 2,000 g for 15 min at 4°C to separate out the plasma fraction. Plasma was stored at −80°C until further analyses. The pituitary was harvested and immediately stored at −70°C for molecular biology experiments.

Enzyme immunoassay (EIA) for androgen and corticosterone

Plasma testosterone levels were analyzed by an EIA method (Testosterone EIA kit, Cayman, USA). The testosterone

EIA assay sensitivity was 25 pg/ml and it showed a cross-reaction with 5α-dihydrotestosterone (21%), 11-keto testosterone (12.4%), and 5β-dihydrotestosterone (10%); since the steroids were not separate, the data are expressed as "androgens" (A). Plasma corticosterone levels were analyzed by an EIA method (Corticosterone EIA kit, Cayman, USA), having a sensitivity of 24 pg/ml.

Radioimmunoassay (RIA) for PRL and GH

Plasma PRL was measured by an RIA employing purified bullfrog (*Rana catesbeiana*) PRL (fPRL) (Yamamoto and Kikuyama, 1981) as standard and radioligand, and its antibody. The radioiodination of fPRL was performed by the lactoperoxidase method (Yamamoto and Kikuyama, 1982). For measuring PRL in the plasma, the antiserum was used at a final dilution of 50,000, at which the antiserum bound about 30% of the label in absence of any unlabeled hormone. Other details of RIA procedures were the same as those used in the fPRL RIA (Yamamoto and Kikuyama, 1982).

Growth hormone in the plasma of *Rana esculenta* was also measured by an RIA using purified bullfrog (*Rana catesbeiana*) GH (fGH) (Kobayashi et al., 1989) as standard and radioligand, and an antiserum to fGH, which was used at a final concentration of 1:50,000. Other details were as described previously (Kobayashi and Kikuyama, 1991). Previously, it was demonstrated (Mosconi et al., 1994b) that the slope of the inhibition curve produced by *Rana esculenta* plasma was parallel to that of the bulfrog (*Rana catesbeiana*) GH and PRL.

RNA isolation

Total RNA from three pituitary glands per group was extracted using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1992). Briefly, pituitary glands were homogenized in a denaturing solution (4M guanidinium thiocyanate, 42 mM sodium citrate, 0.83% N-lauryl-sarcosil, 0.2 mM β-mercaptoethanol); then 1/10 volume of sodium acetate (3M pH 3.5) was added and an extraction with phenol:chloroform:isoamylic alcohol (25:24:1) was performed. After centrifugation at 10,000$\times$g for 20 min, total RNA was extracted from the aqueous phase by precipitation, adding 1 volume of 100% ethanol. Final total RNA concentration was determined by optical density reading at 260 nm, and RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on a denaturing 1.2% agarose gel.

Synthesis of PRL and actin RNA probes

For qualitative and quantitative analysis of f-PRL mRNA expression, heterologous RNA probes were used. The probe for PRL was synthesized using a fragment of 830 bp from bullfrog (*Rana catesbeiana*) PRL cDNA as template, while for actin a 1755 bp fragment of cDNA of *C. pyrrhogaster* was used. The RNA probes were synthesized by "in vitro" antisense transcription using T3 or T7 RNA polymerase by digoxygenin (DIG) labeling kit (Roche).

Qualitative analysis of PRL mRNA

To verify the specificity of heterologous RNA probes for determination of PRL and actin mRNA expression in the green frog (*Rana esculenta*), qualitative analysis of both PRL and actin mRNA expression was made by Northern blot hybridization. Gel was loaded with negative controls: brain (without pituitary), liver, testes, neurointermediate lobe of the pituitary; and positive controls: pituitary "in toto", anterior lobe of the pituitary. The expression of actin mRNA was verified in the same RNA samples.

Ten micrograms of total RNAs from frog tissues were run in an agarose denaturing gel (1.2% agarose; MOPS 1X; 0.41 M formaldehyde). The sample was diluted in loading denaturing buffer (50% formamide; MOPS 1X; 0.41 M formaldehyde) and denatured for 10 min at 65°C before running. The transfer of RNA onto a nylon membrane (Amersham) was performed for 3 h at room temperature using a Turboblotter apparatus (Schleicher Schuell). After transfer, the nucleic acid was linked on the membrane by drying at 80°C for 2 h, followed by 2 min UV crosslinking. If not used immediately, the filter was stored at 4°C.

Northern blot analysis was performed in conditions of maximum stringency. Prehybridization was done in a standard buffer (5 $\times$ SSC; 50% formamide; 0.02% sodium dodecyl sulfate; 0.1% N-lauroylsarcosine; 2% Blocking Reagent for nucleic acid hybridization) for 4 h at 68°C. After prehybridization, the RNA DIG labeled probe, diluted in standard buffer, was added, and overnight hybridization was performed at 68°C. After hybridization, a detection process was carried out with a chemiluminescent detection Kit (Roche).

PRL mRNA quantification

Evaluation of PRL mRNA expression at time 0, 1 week stress, 1 month stress in pituitary glands was performed by Northern blot hybridization. Total RNA extraction was performed on groups of three pituitary glands, and three pools were made for each experimental group. Northern blot analysis was done in accordance with previously described conditions. After PRL mRNA detection, the PRL probe was stripped (two washes of 30 min at 68°C in 50% formamide, 50 mM Tris-HCl pH 8, 0.1% SDS, and the membrane was reprobed under the same conditions by β actin probe. Quantitative analyses of PRL mRNA expression were made by densitometry on the X-ray film resulting from chemiluminescent detection, using β actin mRNA expression to normalize the signal.

Statistical analysis

The results from hormone determinations were analyzed by ANOVA with a statistical software package, Stat View 512+TM (Brain Power Inc., USA). *Post hoc* comparisons were carried out using the Neuman Keuls test. A probability level of 0.05 was taken to indicate a statistical difference between means. Results are expressed as mean $\pm$ SD ($n = 5$) of data. To make the standard curve for PRL mRNA quantification, using densitometric values, a linear regression curve was made by the least-squares method. The absolute values of specific mRNA molecules were extrapolated using a standard curve.

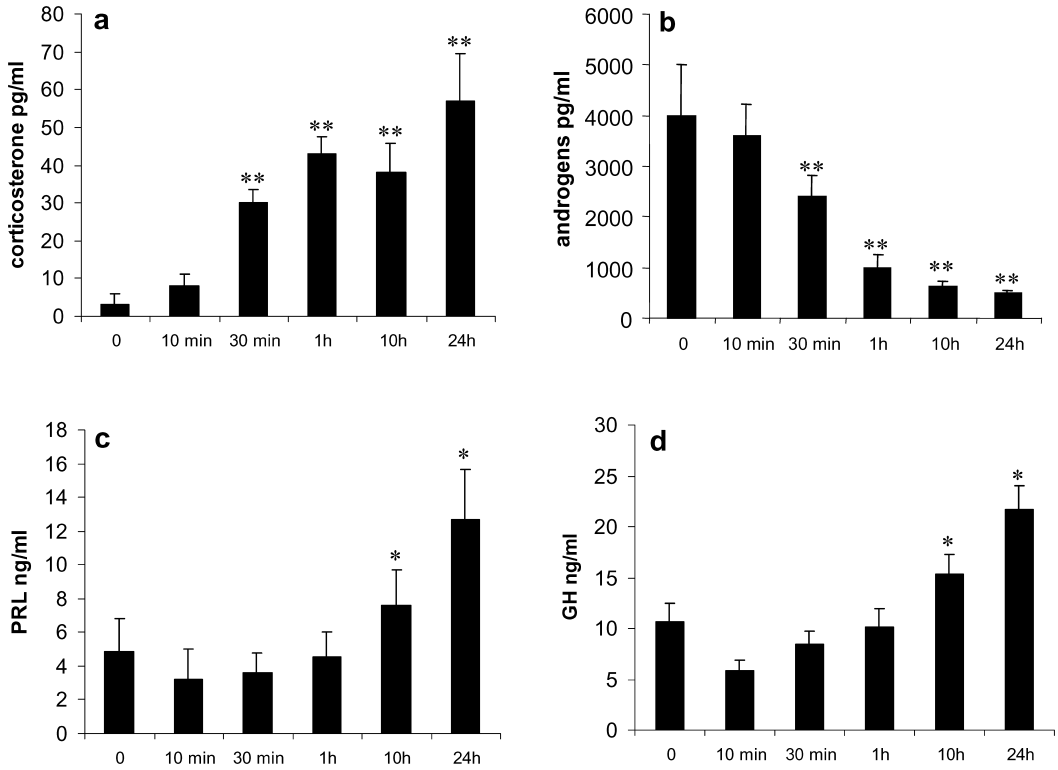

Figure 1. Plasma corticosterone (a), androgens (b), PRL (c), and GH (d) in animals submitted to 24 h of captivity. Results are expressed as mean ± SD of data ($n = 5$). Difference from controls: $^{*}P < 0.05$; $^{**}P < 0.01$.

Results

Short-term captivity stress responses I

In fig. 1, the changes are reported of plasma corticosterone, androgens, prolactin (PRL), and growth hormone (GH) in male frogs sampled after 10 min, 30 min, 1 h, 10 h, and 24 h of captivity. The corticosterone plasma levels were relatively low (5 pg/ml) at time 0 (capture); after 10 min of captivity in laboratory conditions, corticosterone titers increased slightly, the increase being found statistically significant ($P < 0.01$) after 30 min, 1 h, 10 h, and 24 h of captivity (fig. 1a). The androgens trend, shown in fig. 1b, behaved differently compared with that of corticosterone; in the control group, androgen levels were found at 4000 pg/ml, and a statistically significant decrease ($P < 0.01$) was found after 30 min of captivity, which became more consistent after 1, 10, and 24 h.

Regarding the PRL (fig. 1c) and GH (fig. 1d) plasma levels, their changes are consistent with a statistically significant increase ($P < 0.05$) of both hormones after 10 h and 24 h of captivity.

Long-term captivity and salinity stress responses

In these experiments, the effects of long-term captivity combined with salinity stress were evaluated in the pituitary PRL mRNA expression and in the peripheral PRL levels by the sampling performed after 1 week and 1 month of treatment (fig. 2). The hybridizations for both PRL and actin were performed at maximum stringency conditions and gave a single band for the two mRNAs. Moreover, Northern blot analysis of total RNA from several tissues,

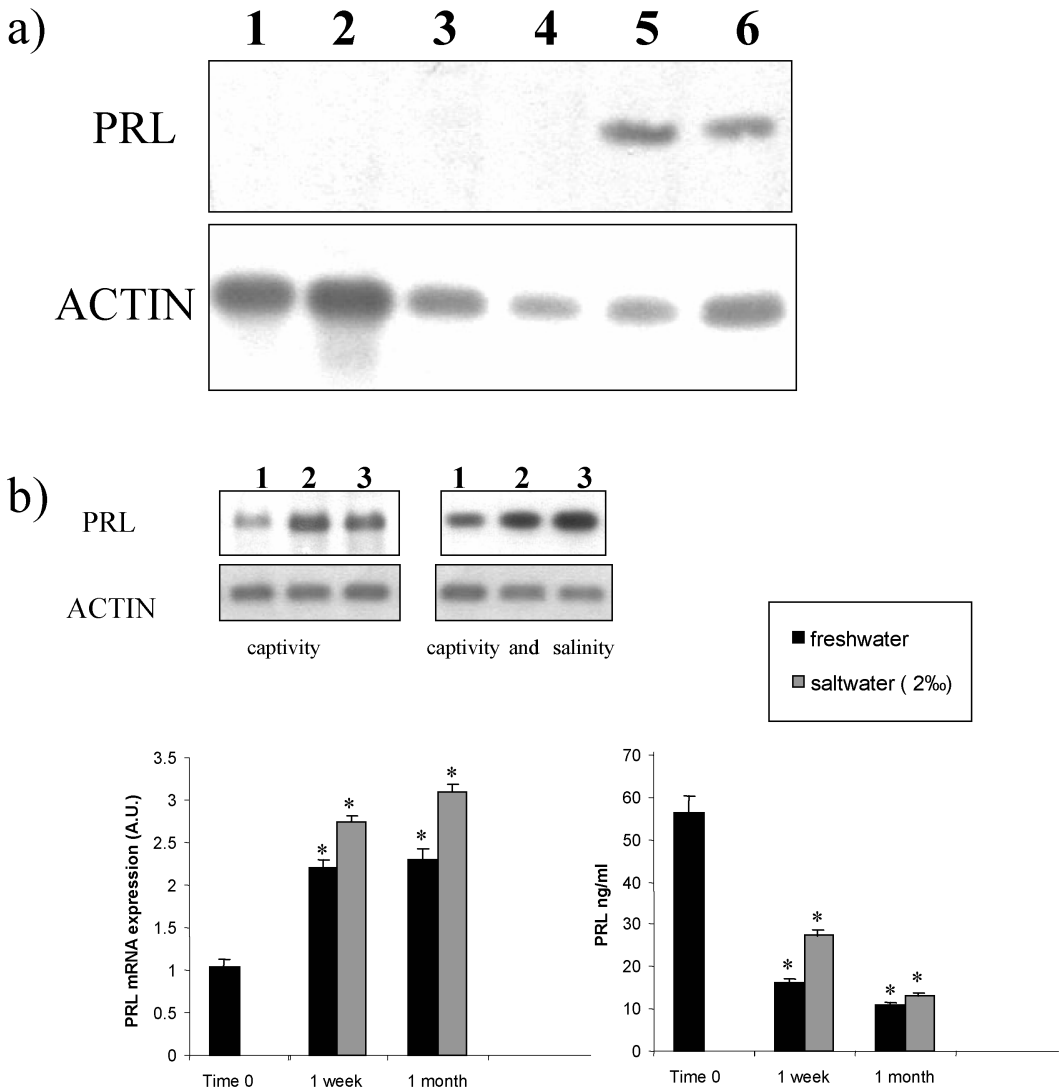

Figure 2. a) Qualitative analysis of PRL mRNA expression made by Northern blot in several frog tissues: 1) brain (without pituitary), 2) liver, 3) testis, 4) neurointermediate lobe of the pituitary, 5) pituitary "in toto", 6) anterior lobe of the pituitary. b) Left. Representative Northern blot of the PRL and actin mRNA in frogs maintained in captivity stress conditions and in frogs maintained in captivity plus salinity stress conditions (1: time 0, 2: 1 week of treatment, 3: 1 month of treatment). Relative tissue concentration of PRL mRNA in the frog pituitary, as determined by semiquantitative Northern blot and densitometric analysis. The PRL mRNA concentrations were normalized using actin mRNA as internal standard, and are expressed as the mean of 3 different experiments ± SD. Right: Plasma PRL level. Difference from time 0: $^{*}P < 0.05$.

showed a positive signal in the pituitary "in toto" and in the pituitary distal lobe (fig. 2a).

Long-term captivity and salinity stress responses were compared with time 0 (capture), and relative variations of PRL mRNA expression showed a statistically ($P < 0.05$) significant increase (fig. 2b). The peripheral PRL behaved very differently since a statistically significant ($P < 0.05$) decrease of PRL titers was evaluated after 1 week and 1 month in both freshwater and saltwater frogs (fig. 2b). No statistical differences were found in plasma corti-

costeroid and androgens in experimental groups compared with control ones.

Discussion

Stress responses in the anuran, *Rana esculenta* were studied by assessing the changes both at peripheral and central level, when different stress paradigms were applied for different times. As found by Licht et al. (1983), short-term (24-h) captivity stress induced activation of the hypothalamus-pituitary-interrenal axis, and the peripheral corticosterone increase provided metabolic support for the initial "alarm phase". The energy demand from stressed animals can also be modulated by the increase of pituitary hormones PRL and GH. In amphibians, it has been found that those hormones are involved in the regulation of growth and differentiation, as well as in reproduction and osmoregulatory function (Mazzi and Vellano, 1987). In addition, in this frog, PRL and GH have been found involved also in the process of vitellogenesis (Polzonetti-Magni et al., 2004).

Short-term stress inhibited peripheral androgens, which follows the idea that stress has negative effects on the reproductive axis. In the control males, androgens levels were found similar to those previously measured in wild animals (Mosconi et al., 1994a), and it seems that short-term captivity stress significantly reduced androgen plasma levels. A high level of corticosteroids negatively influences the gonadal steroids also in fish (Salvante and Williams, 2003), in reptiles (Lance et al., 2004), and in human and mammals (Greenburg and Wingfield, 1987). As previously found in this frog by Mosconi et al. (1994a), the short-term captivity was found a powerful inhibitor of androgen secretion, and in this process the involvement of the opioid system was demonstrated since the androgen inhibition was reversed by treatment with the long-acting opioid antagonist naltrexone. In addition, it has been found that in amphibians the interrenal axis responds also to the stress induced by environmental compounds such as coal combustion waste (Hopkins et al., 1997) and xenobiotic factors; in wild populations, this kind of stress can be related to the deleterious effects of such compounds on the reproductive functions (Mosconi et al., 2002), development, metamorphosis (Kloas et al., 1999; Hayes et al., 2002), and in the immune system responses (Christin et al., 2003); therefore, the stress-related changes depending on ecotoxicological factors should be considered when accounting for the dramatic decline of wild amphibian populations throughout the world.

The results obtained by applying the long-term stress paradigm, in which captivity and salinity stress were combined, suggest that PRL is a very sensitive hormone to captivity and to water salinity. The percentage of water salinity was chosen on the basis of studies performed in *R. esculenta* living in the Lesina lagoon (Mosconi et al., 1997). Previous results showed that short-term captivity induced significant increase of PRL and PRL mRNA (Mosconi et al., 2000), whereas, in the data on the long-term stress responses reported here, both types of stressors activated the PRL gene transcription, perhaps through a long-loop feed-back mechanism depending on decreasing levels of peripheral PRL. It has been extensively demonstrated that in amphibians, dopamine exerts inhibitory effects on PRL secretion; therefore, the hypothesis that long-term stress could activate dopaminergic neurons in the hypothalamus can be considered. Regarding plasma corticosterone and androgens, the values of those hormones after one month of captivity were found to be not significantly different from those measured in the control frogs. As found in fish (Mosconi et al., 1998), in the long-term captivity, neuroendocrine mechanisms, such as the opioid system, may intervene and modulate a kind of recovery and adaptation.

Taken together, the findings reported here suggest that a very sensitive neuroendocrine system is present in this anuran, which responds differently depending on the duration of stress and on the type of stressors.

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