RESEARCH ARTICLE

Overcrowding-Mediated Stress Alters Cell Proliferation in Key Neuroendocrine Areas During Larval Development in *Rhinella arenarum*

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ABSTRACT

Exposure to adverse environmental conditions can elicit a stress response, which results in an increase in endogenous corticosterone levels. In early life stages, it has been thoroughly demonstrated that amphibian larval growth and development is altered as a consequence of chronic stress by interfering with the metamorphic process, however, the underlying mechanisms involved have only been partially disentangled. We examined the effect of intraspecific competition on corticosterone levels during larval development of the toad *Rhinella arenarum* and its ultimate effects on cell proliferation in particular brain areas as well as the pituitary gland. While overcrowding altered the number of proliferating cells in the pituitary gland, hypothalamus, and third ventricle of the brain, no differences were observed in areas which are less associated with neuroendocrine processes, such as the first ventricle of the brain. Apoptosis was increased in hypothalamic regions but not in the pituitary. With regards to pituitary cell populations, thyrotrophs but not somatoatrophs and corticotrophs showed a decrease in the cell number in overcrowded larvae. Our study shows that alterations in growth and development, produced by stress, results from an imbalance in the neuroendocrine systems implicated in orchestrating the timing of metamorphosis. *J. Exp. Zool. 325A:149–157, 2016.* © 2016 Wiley Periodicals, Inc.

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In amphibians, the length of each larval period depends primarily on growth opportunities and individuals respond to their environment either by altering the timing of developmental events or by modifying their morphology, physiology, or behavior (Denver, '98; Gilbert, 2005). Hormones of the neuroendocrine stress axis (hypothalamus-pituitary-adrenal axis) are the principal mediators of physiological and behavioral responses to environmental change (Denver, 2009). Physiological stress in amphibians, as well as in all vertebrates, is usually inferred from an increase in circulating adrenal glucocorticoid hormones (GCs).

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The effects of GCs vary depending on the developmental stage in which a stressor is experienced as well as on the severity of the stressor and also on the subsequent environmental conditions experienced by the animal (Krause et al., 2009; Poore et al., 2010; De Coster et al., 2011).

Several authors have the opinion that a combination of low baseline, fast increase, and rapid induction of negative feedback represents a "good" response. Benefits of the early stages of the adrenocortical stress response—the acute GC response—are presumed to facilitate escape from life-threatening situations such as attacks by predators, inclement weather, or social upheaval (Wingfield et al., 1998; Breuner, 2008). During breeding, many amphibians display a significant elevation of plasma GCs, suggesting that GCs facilitate reproduction at any level (Romero, 2002; Moore and Jessop, 2003). On the other hand, when a noxious stimulus is present for an extended period, it can lead to chronically elevated levels of GCs. Chronic stress can damage health, reproductive function, growth and survival of free-living and captive animals (Glennemeier and Denver, 2002b; Davis and Maerz, 2009; Denver, 2013). Larval phenotypic variation can have long-term fitness consequences for survival and reproduction through altering how animals respond to changing environmental conditions across life stages (Warne and Crespi, 2015). Although models of chronic stress are difficult to find in nature, in recent years, growing evidence has demonstrated that several factors such as pond drying, high larval density and/or food restriction lead to an activation of physiological processes that may serve as mechanisms to cope environmental stressors in amphibian larvae (Crespi and Denver, 2005; Hu et al., 2008; Gomez-Mestre et al., 2013). In particular, Hayes ('97) and Glennemeier and Denver (2002b) measured elevated corticoid content in premetamorphic tadpoles of different anuran species subjected to either low and high larval density and limited food and described what appears to be chronically elevated GC levels.

Chronic stress can also have profound effects on the morphology and functionality of several brain regions (Krishnan and Nestler, 2008). Dagytè et al. (2009) demonstrated that rats exposed to chronic, but not acute, prenatal stress exhibit suppression of cell proliferation and neuronal differentiation in the hippocampus. It has been described that prenatal stress also caused a strong decrease in brain cell proliferation. (Lemaire et al., 2000; Kawamura et al., 2006), and an increase in caspase-3 activity of the litter (Van den Hove et al., 2006). In the rainbow trout, it has been recently suggested that the growth-suppressing effect of chronic cortisol is actually a consequence of food intake reduction, caused by an increase of circulating leptin, together with a reduction in the synthesis of corticotropin releasing hormone (CRH) in the preoptic brain areas (Madison et al., 2015).

In amphibians, little is known about the effect of stress on brain cell proliferation during the development. Hayes ('95) evaluated the effect of corticosterone treatment on different tissues in two species of anuran larvae and demonstrated that the brain and pituitary gland of corticosterone-treated tadpoles exhibited decreased cell density as well as many pyknotic cells. Because of their complex life cycles, amphibians are ideal for investigating environmental effects on early development as well as their impact on future phenotypic expression and fitness (Relyea, 2007). Furthermore, the effect of environmental stress on tadpole growth and development in many ways parallels the effect of perinatal stress on growth and development of mammals (Weinstock, 2001; Hu et al., 2008; Camozzato et al., 2009, Buchholz, 2015). To our knowledge, this is the first study that analyzes the rates of cell proliferation and apoptosis in neuroendocrine areas of amphibian larvae. In this context, we propose to analyze whether the decrease in larval growth and development under stress conditions correlates with a decrease in the rate of cell proliferation in brain areas which are associated with the neuroendocrine control of growth and metamorphosis. Since the balance between cell proliferation and cell death determines the tissue homeostasis, we also analyzed the apoptotic rate in the key brain areas.

MATERIALS AND METHODS

Animals and Treatments

Rhinella arenarum tadpoles were obtained by in vitro fertilization according to Paz et al. ('95). For all experiments, larvae were maintained in dechlorinated tap water with a constant photoperiod and temperature (12:12 hr, 22°C) and fed with boiled chard. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, and the principles of the NIH (publication 8523, revised 1985). To determine the effect of population density on larval development, two experiments were performed.

Experiment 1. Premetamorphic tadpoles at Gosner stage 25 (Gosner, '60) were placed in containers (experimental units) in two different population densities: low density group with five larvae per liter of water (control), and high density group with 40 larvae per liter of water (overcrowded), with seven replicates each, using a random design. Densities were established based on our previous observations on growth and development in *R. arenarum* larvae (Distler et al., 2009). Both densities were maintained in tanks containing 10 L of dechlorinated tap water each. The number of days it took for animals to complete the metamorphic process was registered for each treatment and body weight was determined as an indicator of growth using a precision scale (0.001 g).

Experiment 2. This experiment was conducted as Experiment 1, however, treatment started at Prometamorphic Gosner stage 35.

Tadpoles were anesthetized by the immersion in 0.01% neutralized MS222, weighed, measured its length, and either homogenized and frozen at −70°C for later corticosterone extractions and Radioimmunoassay (RIA) or fixed in Bouiń's solution and embedded in paraffin for immunohistochemical analyses.

Whole-Body Corticosterone Extraction and RIA

Premetamorphic larvae bodies from control and overcowed groups were homogenized in PBS 0.025 M and centrifuged at 15,000*g* for 20 min. Supernatants were extracted three times with 3 mL of cold dichloromethane and the combined organic extracts evaporated under nitrogen pressure. Low polar lipids from the extracts were precipitated by the addition of 1 mL methanol. After 48 hr at 4°C, samples were centrifuged at 1,000*g* for 20 min and the supernatant evaporated under pressurized nitrogen gas, resuspended in 1 mL hexane and placed at 4°C for 24 hr to precipitate high polar lipids. After that, hexane was evaporated and samples were re-dissolved in 100 mM borate buffer, 0.1% gelatin, pH 8.0. Recovery (between 62% and 69%) during the entire procedure were calculated in parallel samples of each stage by adding [^{3}H]-corticosterone. Radioimmunoassay was performed according to Denari and Ceballos (2005). The antiserum against corticosterone was used in a final dilution of 1/22,500. The cross-reactivity with aldosterone was less than 0.1% (Gomez-Sanchez et al., '75). The sensitivity of the assay was 50 pg/mL. Intra and inter-assay coefficients of variation were below 8% and 12%, respectively. The assay was performed using five replicates for control and for overcrowed groups. Corticosterone was assayed in triplicate.

BrdU Incorporation

Stage 31 tadpoles ($n = 5$, for each treatment) were treated by immersion in Phosphate Buffered Saline (PBS) with 10 mM 5-Bromo-2′-deoxyuridine (BrdU, Sigma B5002, St. Louis, MO, USA), for 30 min at room temperature (22°C–24°C) as described by Quick and Serrano (2007). Tadpoles were maintained in dechlorinated tap water for 2 hr after BrdU exposure prior to euthanasia and samples were processed for BrdU incorporation by using immunohistochemical methods.

Immunohistochemistry

After fixation in Bouin's solution (24 hr, 4°C), animals were dehydrated in graded concentrations of ethanol, cleared in xylene and embedded in Histoplast (Biopack, Argentina). Serial sagittal or transversal sections were cut at 7 μm and mounted onto Superfrost Plus (Erie Scientific Co, New Hampshire, USA) slides. Then, sections were deparaffined in xylene, rehydrated through a series of graded alcohols and rinsed in PBS. For activated caspase-3 immunodetection, the protocol described by Jungblut et al. (2010) was followed with minor modifications. Briefly, sections were incubated 10 min in 10 mM sodium citrate buffer, pH 6.0 at sub-boiling temperature. Tissue sections were treated with 3% hydrogen peroxide (H_2O_2) solution to quench endogenous peroxidase activity. Non-specific antigen binding sites were blocked by preincubation with TNB blocking solution (Cat. FP1020, NEN Life Science Products, Boston, MA, USA) and subsequently incubated overnight at 4°C with the primary antibody rabbit anti activated caspase-3, 1/50 (Cell Signaling, Boston, MA, USA). For BrdU-labelled nuclei detection, sections were incubated overnight with mouse anti-BrdU, 1/200 (GE Healthcare, Amersham™, UK) and then treated with the appropriate biotinylated antibody (Jackson Immunoresearch Laboratories, Baltimore, MD, USA) followed by avidin–horseradish peroxidase–biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). The reaction was visualized by exposure to 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining kit (Dako, Carpinteria, CA, USA). The slides were mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Omission of the primary antibody eliminated all staining and served as a negative control. Control slides from animals that did not receive a BrdU injection revealed no staining (data not shown).

For pituitary immunodetection, mouse anti-ACTH 1/400 (Dako, Copenhagen, Denmark), mouse anti-TSH 1/100 (Dako) and rabbit anti-GH 1/100 (Peninsula, Belmont, CA, USA) were used separately and tissue sections were incubated overnight at 4°C. All these antibodies have been previously used and validated in this species (Miranda et al., '95). Slides were processed in the same manner as explained above. For the quantification of pituitary hormones content, the number of positive immunostained cells was counted and relativized to the area of the gland.

Proliferation Analysis

The number of BrdU-positive (BrdU+) nuclei was counted in serial transversal sections of the brain. For the proliferation analysis in the pituitary, the BrdU+ cells were counted in the entire gland and then relativized to a fixed area. Whereas, in the case of the hypothalamus, first and third ventricle the BrdU+ cells were counted along the subventricular zone in four comparable sections for each animal. Because the average diameter of brain cells are approximately 10 μm, we counted BrdU+ cells leaving two sections between each, so there is low probability that labeled nuclei would be counted twice, causing an overestimation of proliferating cells.

Apoptosis Analysis

For activated caspase-3 quantification, serial sagittal sections covering the entire pituitary and hypothalamic regions from each animal were selected and the total of DAB-positive cell was counted. The TUNEL technique (Apoptag plus, Millipore, Billarica, MA, USA) was used to confirm the results obtained with activated caspase-3 (data not shown).

Statistical Analysis

Results were expressed as median ± standard error (SEM) and analyzed with the Infostat software. In all experiments

population density was the explanatory variable with two levels: low density (control) and high density (overcrowded). Response variables in each experiment were compared by performing a Student's *T*-test. The homogeneity of variances within groups was verified with Bartlett's test. In all cases, a value of $P < 0.05$ was considered statistically significant. However, the exact *P*-value obtained in each experiment was informed in the results section.

RESULTS

Growth and Developmental Rates in Response to Intraspecific Competition.

Premetamorphic tadpoles (Experiment 1) were exposed to an intraspecific competitive environment in order to determine the influence of overcrowding in several parameters. Tadpoles exposed to this condition had a slower developmental rate than control animals (34 ± 1 days and 51 ± 7 days, respectively, $P = 0.027$, $n = 10$ for each condition).

Prometamorphic tadpoles (Experiment 2) did not show differences in developmental rate between control and overcrowded groups. In this case, metamorphosis in control and high-density situation took place in 23 ± 8 days and 27 ± 9 days, respectively (Experiment 2, $P = 0.748$, $n = 10$ for each condition). These results show that an adverse environmental condition like overcrowding was more critical for the development of tadpoles at premetamorphosis than it was for prometamorphic larvae, probably due to the requirement of an increasing amount of exogenous nutrients.

As consequence of these results, we decided to carry out our further investigation in premetamorphic tadpoles (Experiment 1). The presence of a high-density environment does not modify the growth rate of premetamorphic larvae as no differences were observed in body length ($P = 0.201$, $n = 10$) or tail length ($P = 0.109$, $n = 10$) (Table 1). However, Table 1 also shows that there was a marked reduction in whole body weight for animals kept in high-density condition. In addition, the time that tadpoles reached stage 31 (beginning of prometamorphosis) was significantly higher than in control animals (Table 1).

Overcrowding as a Stressor Factor in *R. arenarum*

In general, overcrowding is considered a stressful situation and stress is generally associated with an increased concentration of glucocorticoids. In order to determine if a high-density condition activates the hypothalamus-pituitary-adrenal axis, we have determined the whole body content of corticosterone, the glucocorticoid of this species. Table 1 shows that the content of corticosterone was significantly higher in animals kept in high-density condition, confirming that, indeed, overcrowding is a stressful condition.

Analysis of Cell Proliferation and Cell Death as a Consequence of Chronic Stress

It has been proposed that the activation of the hypothalamus-pituitary-adrenal axis and the subsequent elevation in GCs during early life can alter the development of the axis itself (Weinstock, 2001; Welberg et al., 2001). In addition, in this article, we demonstrate that overcrowding produces a retarding effect in tadpoles development and also an increase in corticosterone content. Taking these results into account, we decided to analyze the cellular proliferation balance at different levels such as brain, hypothalamus, and pituitary.

The quantitative analysis of cell proliferation in animals submitted to an overcrowded condition showed a significant reduction in the amount of BrdU+ cells in the pituitary gland (Fig. 1) as well as in the hypothalamus (Fig. 2A, B and G). The same result was observed in the periventricular areas of the third ventricle of the brain (Fig. 2C, D and G) while in the first ventricle the number of BrdU+ cells showed no significant difference between conditions ($P = 0.306$, $n = 5$) (Fig. 2E–G).

The evaluation of apoptosis by using activated caspase-3 immunostaining showed a higher number of apoptotic figures in

Table 1. Parameters measured in Experiment 1.

Determination	Control	Over-crowded
Body length	20.98 ± 0.90 mm	19.48 ± 0.60 mm
Tail length	11.95 ± 0.47 mm	11.08 ± 0.11 mm
Weight	1.114 ± 0.106 g	0.863 ± 0.041 g[a]
Days to stage 31	12.5 ± 1.45 days	24.6 ± 4 days[b]
Corticosterone	56.8 ± 13.01 pg/larvae	276.4 ± 41.03 pg/larvae[c]

Tadpoles in G25 stage were subjected to two densities: Five larvae/liter (Control) and 40 larvae/liter (Overcrowded). Measurements were performed when tadpoles reach stage G31 of development.
[a]Significant difference $P = 0.0416$, $n = 10$.
[b]Significant difference $P = 0.0098$, $n = 5$.
[c]Significant difference $P = 0.0286$, $n = 10$.

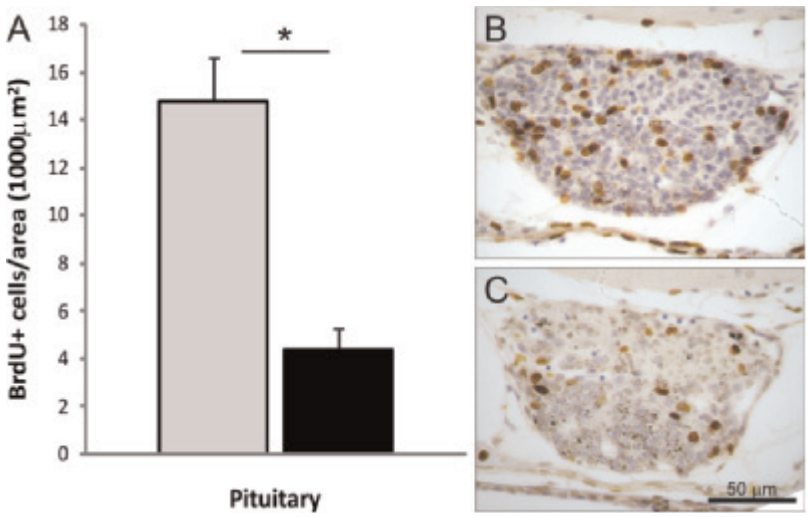

Figure 1. Proliferation analysis in the pituitary gland. (**A**) Animals exposed to overcrowding conditions showed a significant reduction in the number of BrdU positive cells in the pituitary gland (black bar) compared to control animals (gray bar), *significant difference $P = 0.0002$. (**B** and **C**) Representative images of the pituitary (transversal sections, top: dorsal) of control and overcrowded animals, respectively.

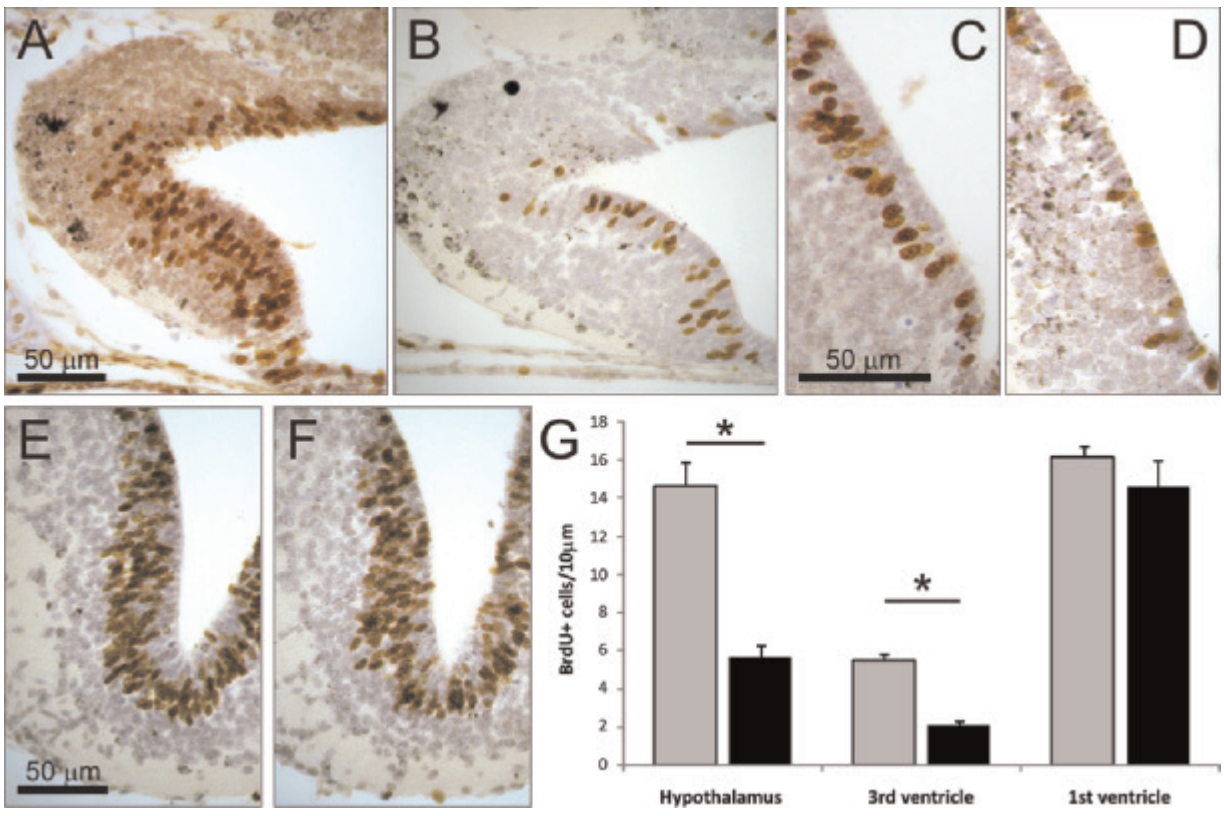

Figure 2. Proliferation analysis in the brain. (**A–F**) shows representative images (transversal sections, top: dorsal, right: medial) of different brain areas: Hypothalamus (**A** and **B**), 3rd ventricle (**C** and **D**), and 1st ventricle (**E** and **F**) of controls (**A**, **C**, and **E**) and overcrowded animals (**B**, **D**, and **F**). (**G**) A significant reduction in the amount of BrdU positive cells was observed in the Hypothalamus and 3rd ventricle (*$P = 0.0001$) but not in the 1st ventricle ($P = 0.305$) of overcrowded animals. Gray bars: control animals, black bars: overcrowded animals.

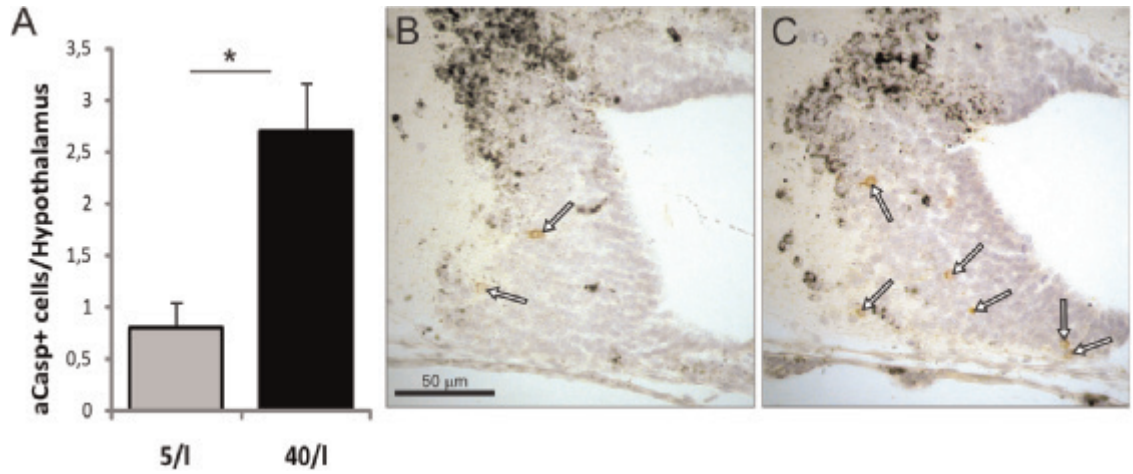

Figure 3. Effects of overcrowding condition on the hypothalamus. (**A**) Quantification analyses showed a higher number of apoptotic cells in the hypothalamus of overcrowded animals (black bar) compared to control (gray bar), *significant difference $P = 0.0054$. (**B** and **C**). Shows representative images of the hypothalamus (sagittal plane, top: dorsal, right: caudal) of controls and overcrowded animals, respectively. Arrows indicate hypothalamic apoptotic cells (activated caspase-3 immunoreactive).

the hypothalamic areas of overcrowded tadpoles than in the same structure of the control group (Fig. 3). On the other hand, no apoptosis was detected in pituitary areas of neither overcrowded or control groups. Similar results were obtained by TUNEL assay (data not shown).

Analysis of Cell Population in the Pituitary Gland

In order to determine if the overcrowded condition produced any change in the number of pituitary cells, immunohistochemistry was performed for detecting tyrotrophs, corticotrophs, and somatotrophs in control and in overcrowded animals. Figure 4 shows that the number of immunopositive ACTH and GH cells did not differ between groups while the number of immunopositive TSH cells was significantly higher in the overcrowded group.

DISCUSSION

All stress responses begin with the central nervous system perceiving a potential threat to homeostasis. It is well known that once perceived by the central nervous system, each stress situation has the potential to trigger different compensatory responses among which the activation of neuroendocrine pathways has a broad, long-lasting effect on the body homeostasis (Moberg and Mench, 2000). Several authors have documented that in amphibians, exposure to adverse conditions elicits a stress response and a resulting increase in endogenous corticosterone levels (Hayes, '97; Glennemeier and Denver, 2002a). As a consequence, exposure to chronic stress could alter larval growth and development, interfering with the metamorphic process (Glennemeier and Denver, 2002a,b). In this work, we conclude that overcrowding of *R. arenarum* tadpoles during the premetamorphic stages provoked a stress response resulting in a significant increase in whole body corticosterone with growth-suppressing effects and delayed metamorphosis. Therefore, overcrowding could be defined as a chronic stress condition, since the animals are exposed to the stressor continuously for an extended period (Li et al., 2015).

In *R. arenarum*, the BrdU incorporation assay indicated a decrease in cell proliferation in several brain areas of chronically stressed animals. This decrease in the number of mitotic images was observed in subventricular area of the brain—third ventricle—as well as in hypothalamic and pituitary level of overcrowded larvae. Our results are in accordance with those reported by Hayes ('95), who showed a reduction in cell density in the brain and pituitary areas upon the exposure of *Bufo boreas* larvae to exogenous corticosterone. However, Hayes ('95) concluded that corticosterone treatment resulted in the presence of pyknotic cells throughout the brain and pituitary gland, suggesting that the decrease in cell density could also be due to an increase in cell death. In our study, there was no evidence of cellular apoptosis in the pituitary of stressed or control animals, while a higher number of apoptotic bodies was observed in the hypothalamus of larvae kept in overcrowded condition. In vertebrates, apoptosis is essential for the maintenance of tissue homeostasis and an accurate development (Danial and Korsmeyer, 2004) and then, an increase of apoptosis in brain areas could constitute an adapting mechanism to different environmental condition. However, under our experimental conditions we cannot discriminate whether the increase of apoptosis constitutes an adapting mechanism or a pathological event.

In mammals, many studies have demonstrated that exposure to a stressful situation during prenatal stages, that is, exposure of

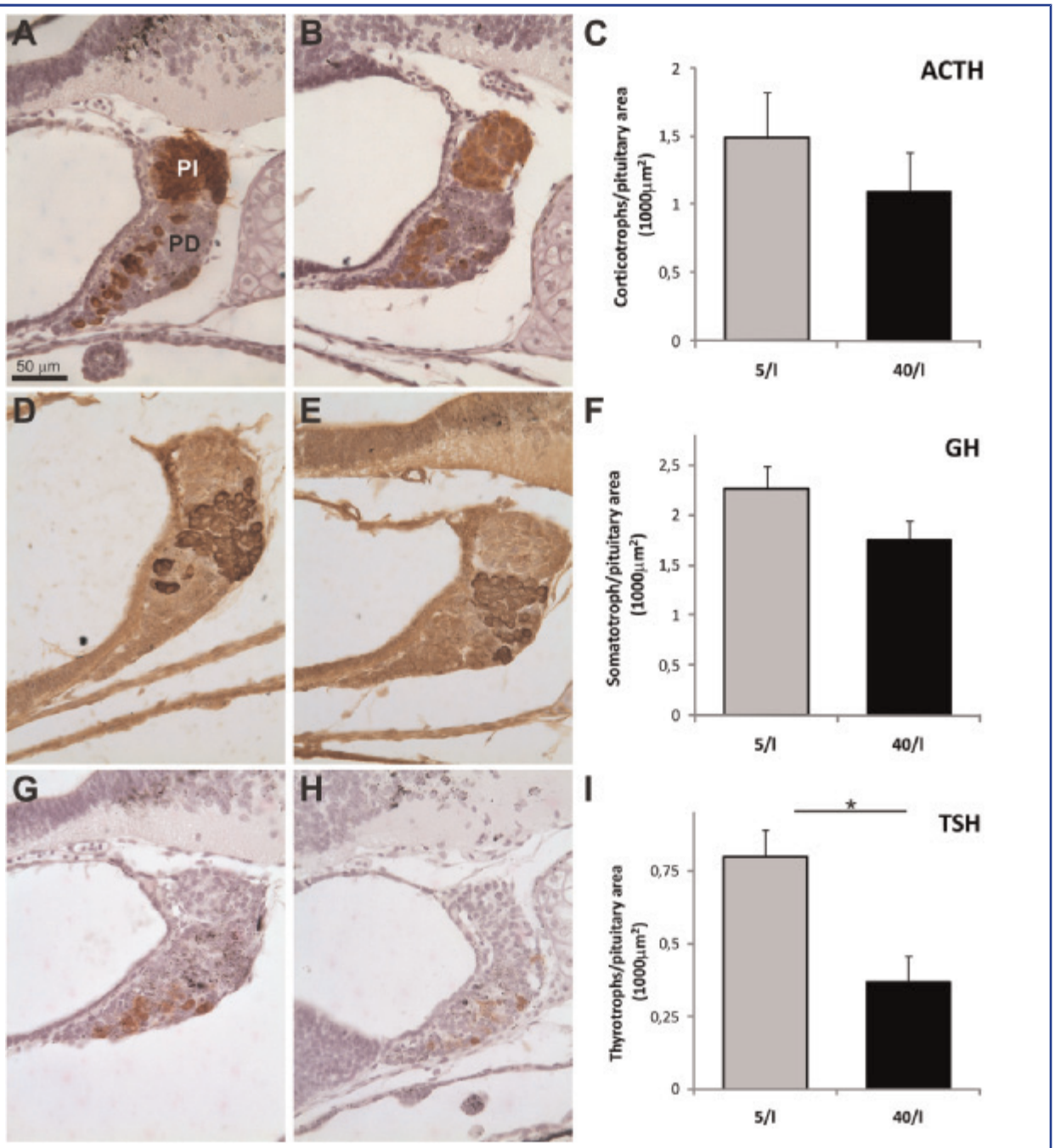

Figure 4. Immunohistochemical analysis of cell populations in the pituitary gland. Panels show representative images (sagittal plane, top: dorsal, right: caudal) of the immunoreaction obtained for corticotrophs (**A** and **B**), somatotrophs (**D** and **E**), and tirotrophs (**G** and **H**) of control (**A**, **D**, and **G**) and overcrowded animals (**B**, **E** and **H**). Overcrowding conditions produced no significant differences in the number of corticotrophs ($P = 0.402$, $n = 5$) and somatotrophs ($P = 0.12$, $n = 5$) compared with control animals (**C** and **F**, respectively), while a significant decrease in the number of TSH immunoreactive cells is observed in overcrowded animals in comparison to control ($P = 0.029$, $n = 5$) (**I**). PD: Pars distalis, PI: Pars intermedia. Scale bar: 50 µm.

pregnant females, provokes in the offspring several responses such us an increase in corticosterone levels, a decrease in newborns body weight and also, modifications in different brain areas development in comparison to control animals. Baquedano et al. (2011) demonstrated that subchronic prenatal stress reduced the rate of cell proliferation in the hippocampus, hypothalamus, and pituitary (HHP) axis in rats, and also inhibited cell death in the same structures, suggesting that rats prenatally exposed to stress show a slowing of the cell cycle in the HHP axis in adult animals. In contrast, in *R. arenarum*, chronic stress was deleterious to the larval growth when it was applied only in premetamorphosis (stage G26–G30), but not in prometamorphosis (stage G31–G41). In prometamorphosis, overcrowding was unable to inhibit tadpole growth and development. Our results are partially in accordance with other authors (for a review see Denver, 2013) since *R. arenarum* shows growth and developmental retardation during premetamorphosis in response to environmental stressful conditions, but in prometamophic stages stress does not accelerate metamorphosis. There seems to be differences in the Hypothalamic–Pituitary axis sensitivity between premetamorphosis and prometamorphosis in *R. arenarum*.

The decrease in cell proliferation observed could be attributable to mechanisms other than glucocorticoids. It has been documented that thyroid hormone (TH) induces DNA replication in the limbs as well as the brain during development of *Xenopus laevis* tadpoles (Das et al., 2006). Also, Denver et al. (2009) analyzed the involvement of thyroid hormone receptors in neural cell proliferation during metamorphosis of *X. laevis* demonstrating that T3 induces cell proliferation in the tadpole brain, predominantly via TRα. Since we observed a reduction in cell proliferation in several areas, as well as slower development in overcrowded tadpoles, we could infer that the stressful condition produces a drop in plasma TH due to the increase of GCs. Although in our study, we have not evaluated whether circulating TH levels are diminished in the overcrowded condition, we observed that there is a decrease in the number of thyrotrophs in these animals. However, more studies are required to elucidate the cellular and molecular levels GCs are acting to modify the development of *R. arenarum* tadpoles.

In conclusion, we have demonstrated that under chronic stress condition, the growth and development of *R. arenarum* larvae is modified. These changes are associated with a decrease in pituitary gland proliferation, which is one important participant in the regulation of metamorphosis. In addition, overcrowding produces an increase in whole body content of corticosterone, this increase being also associated to a decrease in cell proliferation of the third ventricle, hypothalamus, and pituitary without any change in the first ventricle, a decrease in the number of thyrotrophs and an increase in hypothalamic apoptosis. All these areas are deeply involved in the neuroendocrine control of amphibian metamorphosis. All these changes during larval development, as a consequence of overcrowding, can have long-term fitness cost, altering how animals respond to environmental conditions across life stages.

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