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Abstract

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Effect of the Organochlorine Pesticide Endosulfan on GnRH and Gonadotrope Cell Populations in Fish Larvae

Yanina G. Piazza · Matias Pandolfi · Fabiana L. Lo Nostro

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Abstract Endocrine-disrupting chemicals can influence the hypothalamus–pituitary–gonad axis and possibly affect reproduction in vertebrates. We analyzed the effect of 30-day endosulfan (ES) exposure in sexually undifferentiated larvae of the cichlid fish *Cichlasoma dimerus*. The number, area, mean cytoplasmic and nuclear diameter, and mean cytoplasmic optical density of gonadotropin-releasing hormone (GnRH) I, II, and III immunoreactive (ir-) neurons and β follicle-stimulating hormone (βFSH) ir-cells were measured. Animals exposed to the highest ES concentration (0.1 µg/l) showed a decrease in GnRH I nucleus/cytoplasm area ratio on exposure. Nuclear area and mean nuclear diameter of βFSH ir-cells was higher in ES treated fish. βFSH nucleus/cytoplasm area ratio was high in exposed animals, and animals exposed to 0.1 µg/l ES showed smaller mean cytoplasmic optical density. These findings suggest that ES affects GnRH I and βFSH protein synthesis/release. However, these responses seem to be insufficient to affect gonadal differentiation at this stage of development.

The neuroendocrine system of the hypothalamus–pituitary–gonad (HPG) axis regulates reproduction in vertebrates and can be influenced by chemicals, therefore affecting the reproductive system. Neurotoxic environmental contaminants recognized as endocrine-disrupting chemicals (EDCs) have aroused considerable interest in the field of neuroendocrinology (Gore 2000; Pillai et al. 2003; Panzica et al. 2005; Gore 2008a, b). Among these pollutants, organochlorine pesticides are considered to be hazardous because they are very persistent, are nonbiodegradable, and are ubiquitously found in the environment (Palmer and Palmer 1995; Donohoe Re-irradiation in Head and Neck Cancer Curtis 1996). After international recognition of their long-term negative impacts on the global environment, the use of organochlorines in global agriculture has been largely banned (RAP-AL 2008; United Nations 2009). However, endosulfan (ES) remains as one major exception. ES (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3benzo-dioxathiepin-3-oxide) is a cylodiene organochlorine insecticide used for the control of insects and mites in crops of high commercial value (RAP-AL 2008). ES is slightly soluble in water, but it dissolves in most organic solvents (Harding 1979). Given that organic solvents are commonly used in commercial formulations, they might contribute to the overall effect of ES on the dysfunction of the endocrine system (Hutchinson et al. 2006; Mortensen and Arukwe 2006). Exposure of *Thalassoma pavo* to ES decreased feeding behavior related to neuronal degeneration in the mesencephalon and the hypothalamus (Giusi et al. 2005). Cytological and structural oogonia and oocyte malformations, an important decrease in gonadotropin (GtHs) neurosecretory activity, as well as damage of the axons that innerve the pituitary were observed in adults of *Sarotherodon mossambicus* after chronic exposure to ES (Shukla and Pandey 1986). In *Oryzias latipes*, acute exposure to ES caused alterations in development, sexual behavior, and reproductive physiology (Gormley and Teather 2003).

The decapeptide gonadotropin-releasing hormone (GnRH) is mainly synthesized in the central nervous

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system, and its principal function is to induce GtHs release into the bloodstream. It can also act as a neuromodulator and/or neurotransmitter (Kah et al. 2007). In particular, Perciform species express three different forms of GnRH: salmon express GnRH III; sea bream express GnRH I; and chicken express GnRH II (White and Fernald 1993; Pandolfi et al. 2009). Immunoreactive (ir-) GnRH I fibers are abundant in the pituitary, reflecting its primary hipophysiotropic role and showing the strong correlation between GnRH I expression in the brain and gonadal activity (Senthilkumaran et al. 1999). follicle-stimulating hormone (FSH) and (luteinizing hormone (LH) are glycoproteins synthesized in the vertebrate pituitary. These GtHs are expressed at different time points during the reproductive cycle, playing a critical role in the control and regulation of gonadal development, gametogenesis, and gonadal steroidogenesis in teleosts (Pandolfi et al. 2009).

The South American cichlid fish *Cichlasoma dimerus* (Teleostei, Perciformes) is common in quiet, shallow waters of the Paraguay and Paraná river basins (Kullander 1983), including some heavily agricultural areas. This freshwater species is representative of teleosts in the La Plata River basin and relevant to the Argentinean riverine ecosystems (López et al. 2003). It adapts easily to captivity and spawns with a high frequency during 8 months of the year. *C. dimerus* is a gonochoristic fish, with sex differentiation occurring at 42 days postfertilization (dpf) in female fish and at 72 dpf in male fish (Meijide et al. 2005). Both processes coincide with the appearance of ir-GnRH I fibers on the pituitary. GnRH I can be evidenced by immunohistochemistry (IHC) for the first time in larvae at 22 dpf, GnRH II at 5 dp, and GnRH III at 2 dpf (Pandolfi et al. 2002). Ir-βFSH cells are found in the proximal pars distalis (PPD) and pars intermedia (PI) of the pituitary even before gonadal sex differentiation starts (21 dpf); however LH ir-cells, which are found in the PPD, differentiate much later (60 dpf) (Figs. 1, 2) (Pandolfi et al. 2006).

To our knowledge, in Argentina, mean concentrations in surface and ground water of two river basins have been detected (0.97 and 2.0 µg/l of ES I and II isomers, respectively) (Baudino et al. 2003). Data on how ES affects the HPG axis in fish larvae are scarce; therefore, the aim of the present research work was to expound if sublethal exposure of larvae to ES affected morphometric parameters, anatomic localization, time of appearance of GnRHs and/or GtHs cell populations, and gonadal development.

Materials and Methods

Animals

Adults fish of *C. dimerus* captured in Esteros del Riachuelo, Corrientes, Argentina (27°25'S 58°15'W) were held in

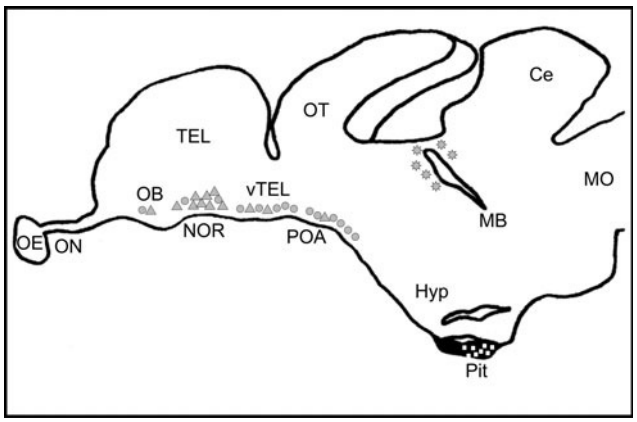

Fig. 1 Sagittal drawing of *C. dimerus* brain showing distribution of GnRH I (*circles*), GnRH II (*stars*), and GnRH III (*triangles*) neurons as well as βFSH (*squares*) pituitary cells. *Ce* cerebellum, *Hyp* hypothalamus, *MO* medulla *oblongata*, *MB* midbrain, *OB* olfactory bulb, *OE* olfactory epithelium, *ON* olfactory nerve, *OT* optic tectum, *Pit* pituitary, *POA* preoptic area, *TEL* telencephalon, *vTEL* ventral telencephalon (modified and reprinted from Pandolfi et al. (2005))

100-L aquaria, with a layer of gravel on the bottom, in filtered tap water at 26°C ± 1°C [pH 7.3] for a 14 to 10-h photoperiod. Fish were allowed to acclimate and were fed daily with a pelleted commercial diet (Tetra food sticks, Germany). Newly hatched larvae (2 dpf) of 2 ± 0.5 mm (total length) were collected from fresh spawns obtained from the breeding couples that were formed within the aquaria. Guidelines on the care and use of fish in research and testing from the Canadian Council on Animal Care (2005) were followed.

Chemicals

The ES used in this study was 94.99% pure (stereoisomer mixtures I and II were 70:30, respectively), assessed by gas chromatography. Exposure concentrations of ES were selected from our preliminary studies (data not shown), and from the 96-hour acute toxicity test, which was determined by probit analysis (LC_{50} at 96 h = 0.3 µg/l for larvae at 10 dpf). All treatment solutions were prepared by adding the necessary stock solution to filtered tap water. To estimate the difference between nominal and actual concentrations of ES caused by errors in the methodology, actual concentrations were measured in water samples from 0.03 and 0.1 µg/l ES aquaria 15 min after each renewal by gas chromatograph-electron capture detector (Environmental Protection Agency SW846 M8081A). The concentrations measured were of 0.02 (for 0.03) and 0.08 (for 0.1) µg/l; therefore, the preparation of the ES stock solution and the dilution in aquaria were considered accurate. ES was dissolved in acetone (0.001%) to produce a 0.01 mg/ml stock solution and stored in the dark at 4°C. New stock solution was prepared before every media renewal.

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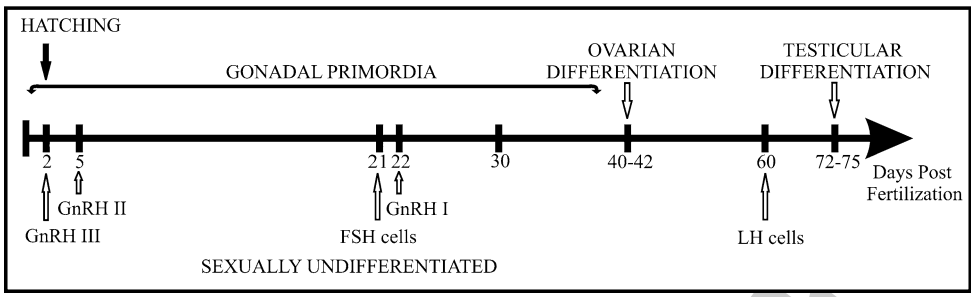

Fig. 2 Schematic time line showing *C. dimerus* ontogenetic development as well as the ontogeny of its gonadotropin-releasing hormone (GnRH I, II, and III) and gonadotropin (FSH and LH) systems at 26.5°C

Sublethal Assays

Larvae (2 dpf) were exposed to ES nominal concentrations of 0.03 and 0.1 µg/l by way of immersion under semistatic conditions. Each concentration was tested in duplicate with 10 individuals per test group in 10-L glass tanks. Control treatments (with and without vehicle) were also performed in duplicate. Acetone was added to the control test group in an amount equal to that present in the highest concentration of ES employed. Media was renewed every 48 h. Larvae mortality, abnormal behavior (loss of balance, muscle tremors, hyperactivity and hypoactivity), and gross anatomic abnormalities (lordosis, body swelling, depigmentation) were observed and recorded throughout the experiment. Whenever larvae were fed, their behavior was observed and recorded. Although no quantitative analyses were performed, larvae abnormal behavior was assessed by qualitative observations, which were always performed by the same observer, at the same time of day (noon), and for the same length of time (15 min).

As soon as larvae started to swim (8 dpf), they were fed with *Artemia* sp. nauplii twice a day. After a 28-day exposure period (30 dpf), fish were weighed (mg); their standard length was measured (mm); and they were killed by decapitation under anaesthesia (fish calmer [Jungle Hypno]).

Tissue Fixation

Larvae heads and trunks were fixed in Bouin's solution for 24 h at 4°C. Samples were then dehydrated and embedded in paraplast (Fisherbrand, WA). Heads were transversally sectioned at 10 µm for immunohistochemical techniques, and trunks were transversally sectioned at 7 µm and stained with hematoxilin and eosin. Gonad external morphology, general tissue organization, and cell cytoarchitecture were analysed.

Single-Label IHC

The immunohistochemical technique was performed according to protocols already tested in this species (Pandolfi et al. 2005, 2006). Briefly, sections were deparaffinized in xylene, rehydrated through a series of graded ethanol to phosphate-buffered saline (PBS; 0.05 M [pH 7.4]) and treated for 5 min with 3% H_2O_2 at room temperature (RT) for endoperoxidase blocking. All sections were then treated for 30 min with PBS containing 5% nonfat dry milk at RT, incubated in a closed moist chamber with their specific primary antiserum overnight at 4°C, washed in PBS, and finally incubated for 45 min with their complementary secondary biotinilated antibody (Vector) at RT. Amplification of the signal for GnRH cell detection was performed using a tyramide based-signal amplification kit (CSA-Peroxidase Kit; Dako) according to the manufacturer's instructions. For ir-βFSH cells, amplification of the signal was achieved by incubation with peroxidase-conjugated streptavidin (Dako), which had been diluted to 1:500, for 1 h. In all cases, peroxidase activity was visualized with 0.1% 3,3′-diaminobenzidine in TRIS buffer (pH 7.6) and 0.03% H_2O_2. Sections were lightly counterstained with hematoxylin, mounted, examined with a NIKON Microphot FX microscope, and digitally photographed (Coolpix 4500; Nikon).

For the precise location of the various GnRH cells and projections, we relied on the detailed atlases of two other perciform species, *Dicentrarchus labrax* (Cerdá-Reverter et al. 2001a, 2001b) and *Haplochromis (Astatotilapia) burtoni* (Fernald and Shelton 1985) as well as previous studies on GnRH neurons localization in this species (Pandolfi et al. 2002, 2005). In contrast, for the precise location of the ir-βFSH cells, we relied on the detailed study performed by Pandolfi et al. (2006) in this species.

Antisera and Specificity Controls

The primary antisera used in this study are listed in Table 1. To confirm specificity of the immunostaining, control sections were incubated with the primary antisera (in their work dilution), which was preabsorbed with an excess of its respective antigen (100 ng/µl). To avoid false-positive results caused by the IHC itself, replacement of primary antisera with PBS and omission of secondary antisera were also performed.

GnRH neurons can be easily and accurately detected with antibodies developed against each GnRH-associated peptide (GAP), which show a greater specificity than using antibodies against the smaller GnRH molecules themselves.

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Table 1 Characteristics of the primary antisera used in the immunohistochemical techniques and work dilution

1° Antibody	Source	Dilution	Raised in
Antis-bGAP (GnRH I)	*D. labrax*[a]	1:600	Guinea pig
Anti-cIIGAP (GnRH II)	*D. labrax*[a]	1:600	Guinea pig
Anti-sGAP (GnRH III)	*D. labrax*[a]	1:600	Guinea pig
Anti-βFSH	*F. heteroclitus*[b]	1:1000	Rabbit

[a] Donated by Dr. José Antonio Muñóz Cueto, Departamento de Biología, Facultad de Ciencias del Mar y Ambientales. Universidad de Cádiz, Puerto Real, España

[b] Donated by Dr. Akio Shimizu, National Research Institute of Fisheries Science, Fisheries Research Agency, Kanazawa, Yokohama, Japan

These GAPs colocalize with each GnRH-expressing cell, thus avoiding cross-reactivity (Ronchi et al. 1992; Polkowska and Przekop 1993; Gonzalez-Martinez et al. 2002; Pandolfi et al. 2005). The generation and characterization of the antisera against βFSH of the cyprinodontiform *Fundulus heteroclitus* were previously described by Shimizu and Yamashita (2002). These antisera were specifically raised against conservative sites of teleost GtHs. For this study, anti-Fh (50 to 60) βFSH was used.

Epitope Unmasking

In the case of ir-βFSH cells, pituitary sections were treated for epitope unmasking after endoperoxidase blocking to enhance antigen immunoreactivity (Shimizu et al. 2003). Sections were placed in an epitope-unmasking solution (Target Unmasking Fluid; Sanbio B. V., Netherlands) for 10 min at 90°C, cooled at RT, and finally washed in distilled water.

Morphometrical Analysis of GnRH- and βFSH-Producing Cells

Although cell bodies are highly irregular in shape, and most are split during histological sectioning, we estimated the number of cells by counting them only when the nucleus was evident in consecutive slides. Because brains were sectioned at 10-µm intervals, cells > 10 µm were counted only if their nucleus was clearly visible. The number of cells was expressed per number of slides that had positive immunostaining. For each fish, 5 to 10 randomly chosen cells were measured. Two-dimensional area (µm^2) and mean diameter (µm) were measured in those ir-cells whose perimeter was clearly discernible in the plane of the section. The outline of the cytoplasm and nucleus of stained cells was traced by moving the mouse-controlled cursor along the digitalized image. The cell area and optical density of the immunostain were analysed using an image processing program (Image pro-plus 4.5 software; Media Cybernetics) (modified from Parhar et al. 2001). Ir-GnRH and ir-βFSH cells were quantified and measured in the same brain regions for all fish. Settings (light intensity, opening of the condenser, *etc*.) of the microscope and the camera were maintained constant. The average optical density of the immunostain per cell was calculated from isolated ir-cells. To decrease variability in the immunohistochemical results, all parameters were controlled and kept homogenous (*e.g.*, control, solvent and treated slides were incubated simultaneously; and time of counterstaining and development were kept constant).

Statistical Analysis

The results were statistically analyzed using one-way or nested analysis of variance (ANOVA) followed by Tukey's post-hoc analysis (Statistica 7.0) whenever significant differences were found. When data did not meet the ANOVA assumptions (homogeneity of variance and normality), it was log-transformed before analysis. Nonparametric analysis (Kruskal–Wallis test) was applied when data could not be assumed to be normally distributed. Values were considered significantly different at $p < 0.05$. In all instances data are presented as mean ± SEM.

Results

Survival and Growth

Larvae survival (mortality) was not affected by acetone or ES exposure (Table 2; Kruskal–Wallis, $p > 0.5$). Weight and standard length of larvae did not differ between treatments at the end of the experiment (Table 2, Kruskal–Wallis, $p \geq 0.5$). Animals exposed to both ES concentrations

Table 2 Larvae mortality, total body weight (mg), and standard length (mm) at the end of the experiment in the different treatments

Treatment	*n*	Mortality (%)	Total body weight ± SEM (mg)	Standard length ± SEM (mm)
Control	8	60	12.7 ± 1.3	7.4 ± 0.2
Vehicle	8	60	10.2 ± 0.1	6.7 ± 0.5
0.03 µg/l ES	7	65	9 ± 1.7	6.4 ± 0.4
0.1 µg/l ES	7	65	8 ± 0.5	6.4 ± 0.2

No statistical differences were observed between any of the parameters (Kruskal–Wallis $p > 0.5$)

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showed hypoactivity, but no external abnormalities were observed in fish from any treatment.

Sex Determination

All animals were sexually undifferentiated at the end of the experiment (30 dpf). There were no detectable macroscopic or microscopic alterations on the gonads of the animals exposed to the vehicle or to the different concentrations of ES (Fig. 3).

Morphometrical Analysis of GnRH- and βFSH-Producing Cells

GnRH and βFSH ir-cells presented a brownish cytoplasm and an unstained nucleus after the immunohistochemical reaction. Ir-GnRH I cells were mainly found in the ventral telencephalon and preoptic areas, ir-GnRH II cells in the midbrain tegmentum, and ir-GnRH III cells in the nucleus olfacto retinalis (NOR). Ir-βFSH cells were found in the pituitary gland (PPD and ventral border of the PI). No differences were found in the anatomic localization and ontogeny of these cells between treatments. The morphology of the different GnRH neurons and βFSH cells is shown in Figures 4 and 5.

GnRH I Neurons

No difference was observed between treatments in the mean number of ir-GnRH I neurons per slide (Kruskal–Wallis test, $p > 0.6$) (Table 3). Animals exposed to 0.1 µg/l ES showed the smallest nucleus/cytoplasm area ratio (Nested ANOVA, $p < 0.05$, Tukey's test, $p < 0.001$) (Fig. 6c). Fish exposed to 0.03 µg/l ES showed a tendency to exhibit higher values of mean cytoplasmic optical density (Fig. 7c). No significant differences were observed for any of the remaining parameters.

GnRH II Neurons

No difference was observed between treatments in mean number of ir-GnRH II neurons per slide (Kruskal–Wallis test, $p > 0.6$) (Table 3). In addition, no difference was found between treatments in cell area, mean cell diameter, nuclear area, and mean nuclear diameter (Nested ANOVA, $p \geq 0.5$); however, fish exposed to 0.03 µg/l ES showed a tendency to exhibit higher values of mean nuclear diameter (Figs. 6, 7a, b). The nucleus/cytoplasm area ratio and mean cytoplasmic optical density showed no differences between treatments (nested ANOVA, $p > 0.5$ and $p \geq 0.05$, respectively) (Figs. 6c, 7c).

GnRH III Neurons

Mean number (Kruskal–Wallis test, $p > 0.9$) (Table 3); cell area, mean cell diameter, nuclear area, mean nuclear diameter (nested ANOVA, $p \geq 0.3$; Figs. 6, 7a, b), nucleus/cytoplasm area ratio, and mean cytoplasmic optical density (nested ANOVA, $p > 0.5$ and $p \geq 0.05$,

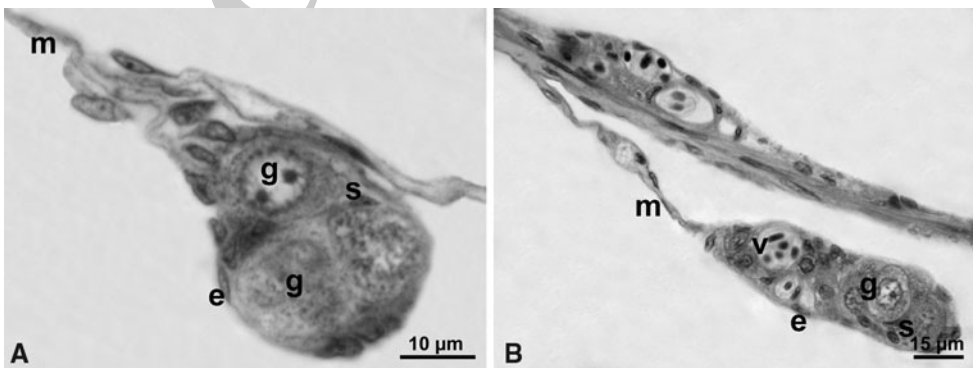

Fig. 3 Cross-section of undifferentiated gonads from (**a**) control (*scale bar* 10 µm) and **b** 0.1 µg/l ES-exposed animals (*scale bar* 15 µm). *E* epithelial cells, *g* gonia, *m* mesentery; *s* support cells, *v* blood vessel

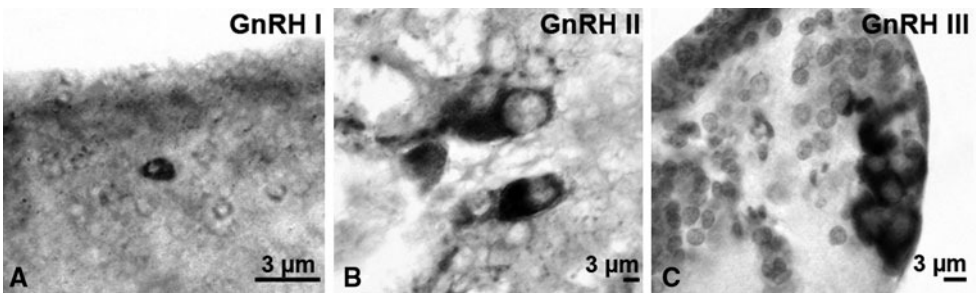

Fig. 4 Photomicrographs of different GnRH neuron populations from control animals. **a** GnRH I located in the preoptic area. **b** GnRH II in the midbrain tegmentum. **c** GnRH III in the NOR. *Scale bars* = 3 µm

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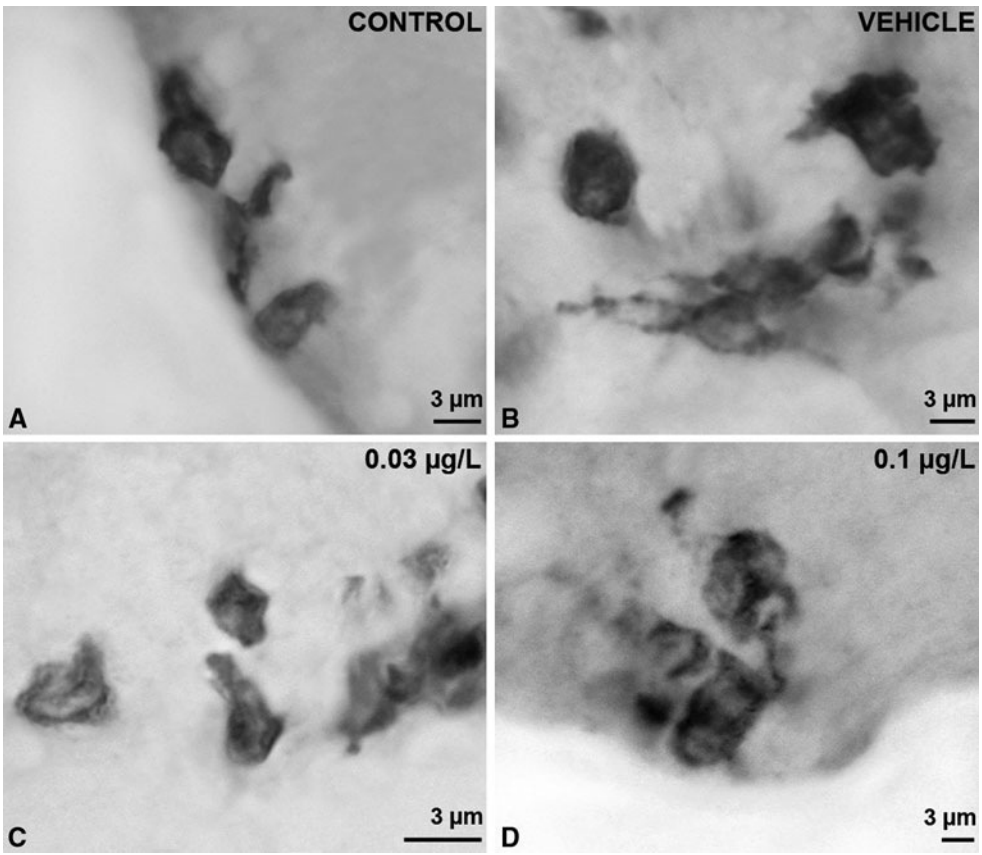

Fig. 5 Photomicrographs of βFSH pituitary cells belonging to: **a** controls, **b** vehicle, **c** 0.03 µg/l, and **d** 0.1 µg/l ES-exposed animals. *Scale bars* = 3 µm

Table 3 Mean cell number of GnRH I, II, and III and of βFSH per slide in control and treated animals

Treatment	GnRH I			GnRH II			GnRH III			βFSH		
	n	Cells/slide ± SEM	Range	*n*	Cells/slide ± SEM	Range	*n*	Cell/slide ± SEM	Range	*n*	Cell/slide± SEM	Range
Control	5	3 ± 0.8	1.6–3.5	4	3 ± 0.9	1.8–3.5	8	18 ± 10	7–38	4	16 ± 5	9–21
Vehicle	5	3 ± 1	2.3–5.5	5	3 ± 0.9	1.8–4.3	8	11 ± 5	6–20	5	26 ± 10	15–39
0.03 µg/l	5	2 ± 0.6	1.4–2.9	4	2.3 ± 1	1–3.5	7	12 ± 6	5–21	5	30 ± 10	17–39
0.1 µg/l	6	3 ± 0.7	1.9–3.7	4	3.5 ± 1.6	2–5.5	7	14 ± 7	5–26	4	22 ± 10	8–32

Kruskal–Wallis test $p > 0.6$

n Number of animals

respectively; Figs. 6c, 7c) showed no difference between treatments.

βFSH Cells

No difference was observed between treatments in the mean number of ir-βFSH cells per slide (Kruskal–Wallis test, $p > 0.6$) (Table 3). Larvae exposed to 0.1 µg/l ES exhibited the highest values of nuclear area and of mean nuclear diameter (nested ANOVA $p < 0.05$, Tukey's test $p < 0.05$) (Figs. 6b, 7b). A concentration-dependent tendency toward the increase of these parameters was observed. The nucleus/cytoplasm area ratio was smaller in control than in acetone and ES-exposed animals (nested ANOVA $p < 0.05$, Tukey's test $p < 0.05$) (Fig. 6c). Fish exposed to the highest ES concentration showed the lowest mean cell optical density of staining; animals exposed to vehicle and 0.03 µg/l ES also showed a lower mean cell optical density of staining than control animals (nested ANOVA, $p < 0.05$; Tukey's test, $p < 0.0001$) (Fig. 7c). No differences were observed for any of the remaining parameters.

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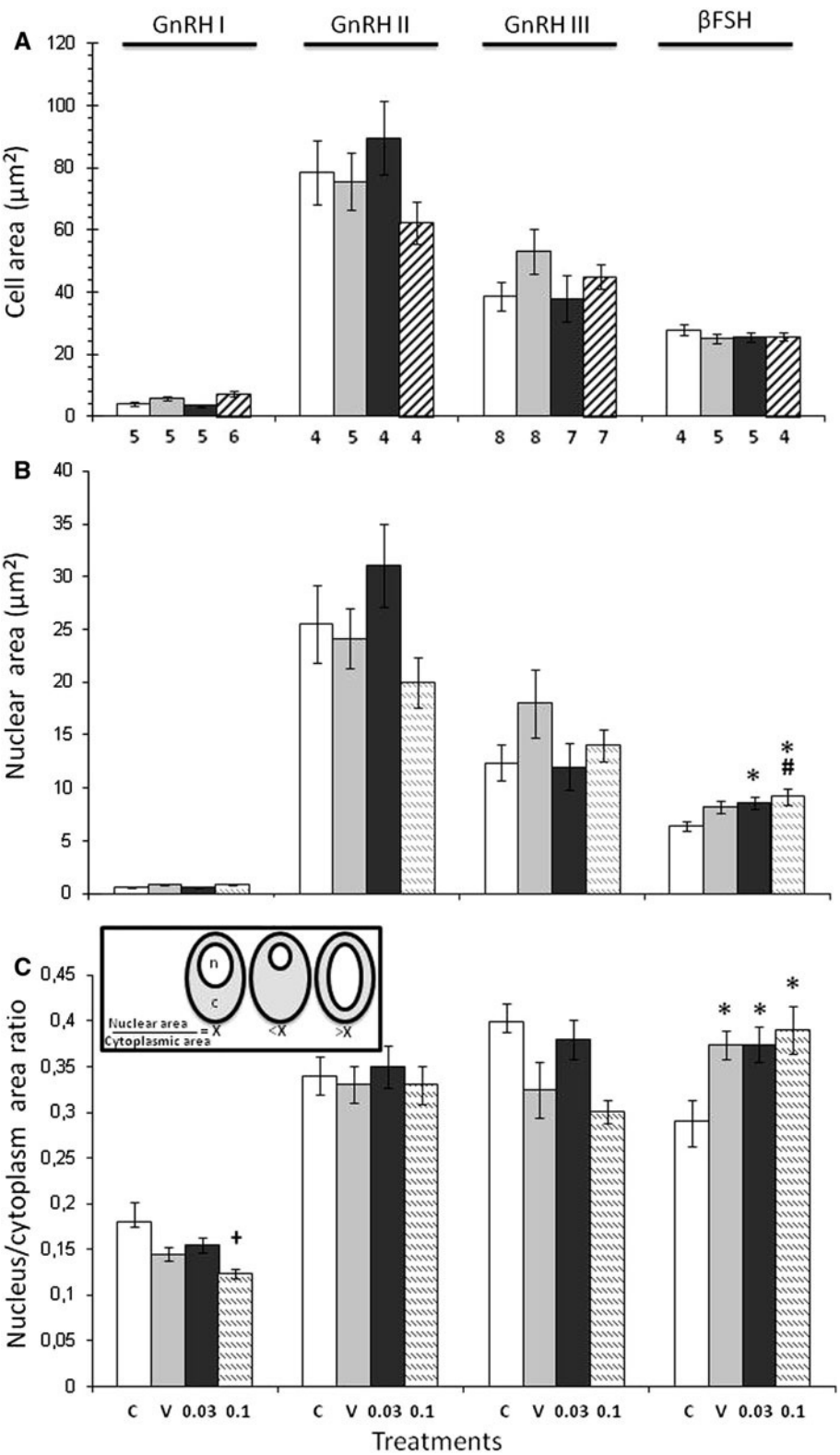

Fig. 6 **a** Cell area, **b** nuclear area, and **c** nucleus/cytoplasm area ratio of GnRH I, II, and III ir-neurons and *β*FSH ir-cells. *C* control, *v* vehicle control; 0.03 = 0.03 µg/l ES; 0.1 = 0.1 µg/l ES. *Bars* show mean ± SEM. *Numbers* indicate sample size for each group. * Significant difference between treatments and control. # Significant difference between vehicle treated and 0.1 µg/l ES. + Significant difference between 0.1 µg/l ES and all other treatments. *Inset* schematic drawing showing the nucleus/cytoplasm area ratio change in accordance with nuclear size. Values were considered significantly different when $p < 0.05$ (nested ANOVA, Tukey's test)

Discussion

In the present study, we examined the effect of long-term exposure to technical-grade ES in larvae of *C. dimerus* before gonadal sex differentiation. There are several short-term studies on how ES affects fry, siblings, or larvae in different ways (Gopal et al. 1981; Shafiei and Costa 1990; Willey and Krone 2001; Gormley and Teather 2003;

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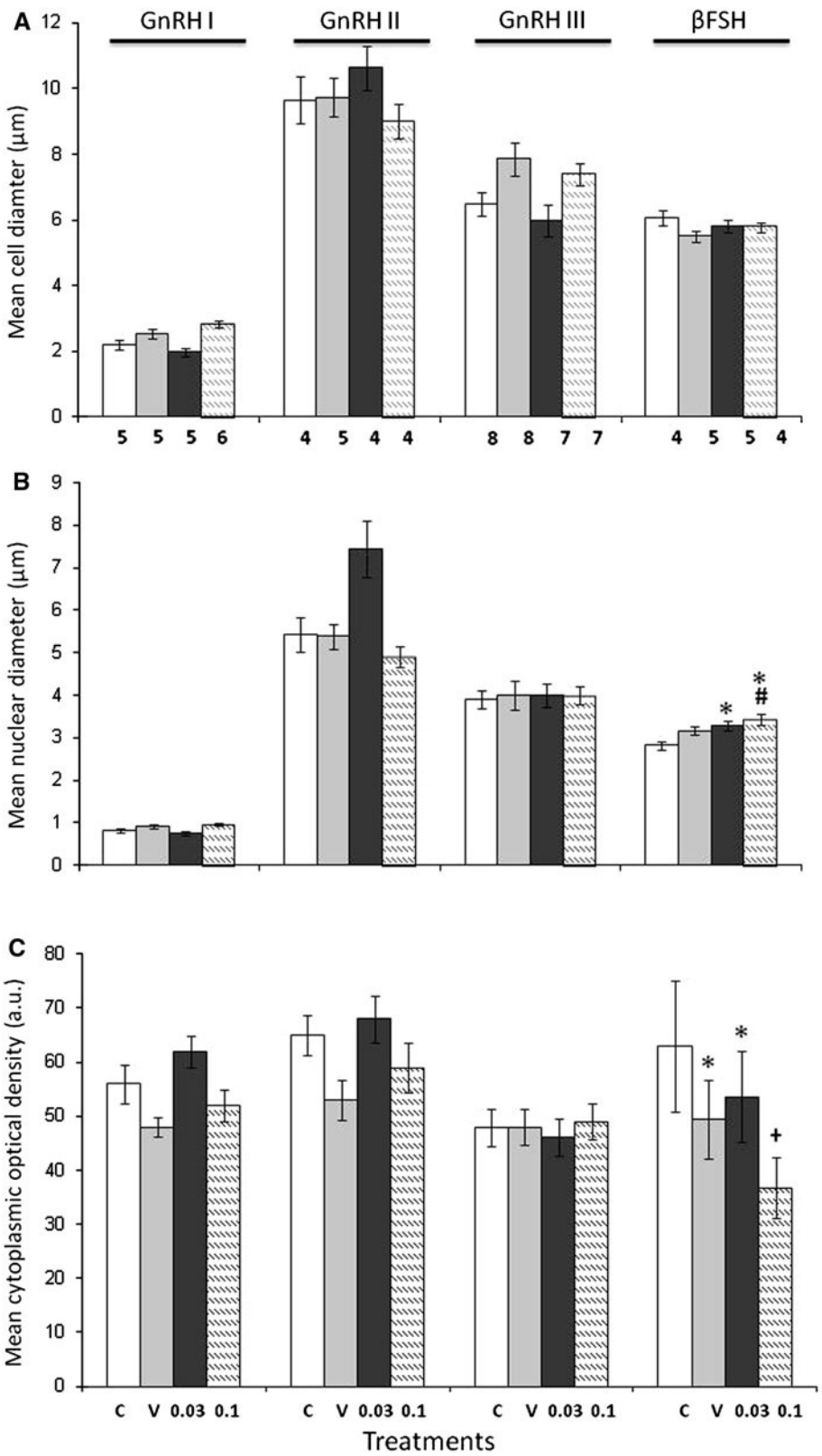

Fig. 7 **a** Mean cell diameter, **b** mean nuclear diameter, and **c** mean cytoplasm optical density of GnRH I, II, and III ir-neurons and *β*FSH ir-cells. *C* control, *v* vehicle control; 0.03 = 0.03 µg/l; 0.1 = 0.1 µg/l ES. *Bars* show mean ± SEM. *Numbers* indicate sample size for each group. * Significant difference between treatments and control. # Significant difference between vehicle treated and 0.1 µg/l ES. + Significant difference between 0.1 µg/l ES and all other treatments. Values were considered significantly different when $p < 0.05$ (nested ANOVA, Tukey's test)

Balasubramani and Pandian 2008; Stanley et al. 2009), however, data on long-term exposures in larvae are scarce. To our knowledge, this is one of the first experimental designs in which a long-term exposure (28 days) to ES, use of fish larvae (30 dpf), and neuroendocrine analysis were combined.

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Abnormal behavior and feeding difficulties were observed after sublethal ES exposure in adults of *T. pavo* (Giusi et al. 2005) and in eggs and fries of *O. latipes* (Gormley and Teather 2003) and after diazinon (an organophosphate pesticide) exposure in adults of *Oncorhynchus tshawytscha* (Scholz et al. 2000). As a result, these behavioral abnormalities could cause weight loss and decreased body size. Sublethal ES exposure caused hyperactivity in adults of *Cyprinus carpio* and hypoactivity in *C. dimerus* larvae; however, none of them showed differences in body weight or size between control and treated animals (Salvo et al. 2008). Therefore, long-term exposure to sublethal concentrations of ES, at least in our species, would not affect larvae and juveniles' early body growth and development.

Larvae mortality was not affected by acetone or ES exposure; however, the high mortality rate observed was expected due to the natural population decrease during the first month of life.

Gonads are sensitive to environmental stimuli around the time of sex differentiation, and they could possibly be affected by EDCs (Nakamura 2000). These chemicals can act during critical periods early in development, thus inducing permanent morphologic changes (Guillette et al. 1995). According to Willey and Krone (2001), *Danio rerio* embryos exposed to sublethal concentrations of ES showed alterations in the distribution of primordial germ cells along the anterior–posterior axis. Exposure to both sublethal concentrations of ES assessed in this study did not cause any abnormalities in the early formation or in the morphology of already-developed gonads. Moreover, as reported for this species under laboratory conditions, gonads were not differentiated at 30 dpf (Meijide et al. 2005). This could suggest that exposure to ES and/or acetone does not accelerate differentiation, at least at the concentrations tested; however, to verify this, exposure during the embryo stage and during and after the sexual differentiation period should be performed.

EDCs can alter plasmatic levels of sex steroids in fish by affecting the hypothalamic and/or pituitary hormone synthesis/secretion (Gore et al. 2008a, b). Hormonal interplay between the brain and the gonads is essential in the sexual differentiation of some fish species (Pandolfi et al. 2006). Environmental cues are perceived and interpreted by the brain, which is involved in both stimulatory and inhibitory regulation of GtHs release from the pituitary gland (Peter et al. 1991). GtHs secretion is regulated by GnRH, and after their release they stimulate steroid production in the gonads. These hormones, at brain levels, stimulate or inhibit further steroid production (Kime 1999). In *T. pavo*, several neurodegenerative events in the diencephalic and preoptic areas of the hypothalamus were observed after exposure to ES (Giusi et al. 2005). These regions are sensitive to pesticides, possibly affecting those cells related to the HPG circuits, with consequences in the reproductive and hormone-releasing activities (Cooper et al. 2000; Bloomquist 2003). In our study, we did not find any abnormalities in the anatomic localization or time of appearance, not only of GnRH neuronal populations but also pituitary βFSH endocrine cells, on exposed animals. Furthermore, we did not find any cytological deformities or abnormalities in GnRHs or βFSH ir-cells. However, in *S. mossambicus*, ES subchronic exposure caused vacuolated and granulated cytoplasms in gonadotropes and thyrotropes, showing size enlargement, and damaged nuclei were also found in a few fish. Moreover, in the same species, exposure to DDT (dichlorodiphenyltrichloroethane), γ-BHC (dehydrochlorination of γ-hexachlorocyclohexane by γ-BHC-assimilating *Pseudomonas paucimobilis*), and malathion produced size enlargement of pituitary cells (Shukla and Pandey 1984, 1986).

Our morphometric studies showed a decreased nucleus/cytoplasm area ratio for GnRH I in animals exposed to the highest ES concentration. This decrease might be due to enlargement of the cytoplasm, possibly by accumulation of the neuropeptide. βFSH ir-cells had a significant increase of nuclear size, mean nuclear diameter, and nucleus/cytoplasm area ratio in 0.1 µg/l ES-treated animals. These results suggest that these cells are more active than the same cells in vehicle and control animals due to enlargement of the nucleus. Cells corresponding to the animals exposed to 0.1 µg/l ES also exhibited a significant decrease in mean cytoplasmic optical density. Taking into account all of these findings, we propose that FSH cells of animals exposed to the highest ES concentration may not only be synthesizing more βFSH protein but also possibly releasing most of their content as well. At this time of gonadal development, the increase in βFSH would not influence their differentiation timing, as was shown in vitro during the sexual differentiating period in this species. In this study, only FSH cells were analyzed because LH cells are not differentiated at this stage of development (Pandolfi et al. 2006). Studies in *Hepteropneustes fossilis* after exposure to different pesticides showed a decrease of GtH secretion and also decrease of a GnRH-like factor level in the hypothalamus, thereby inhibiting synthesis and release of GtH (Singh and Singh 1982). The increase in FSH release does not accelerate sexual differentiation, probably because undifferentiated gonads are not ready yet to respond to FSH and because at this stage there is no evidence of esteroidogenic activity (Meijide et al. 2005). However, we cannot discard a possible endocrine disruption at different levels due to this precocious release of βFSH.

A critical observation in the present study is the fact that the carrier vehicle (acetone) had an effect on βFSH ir-cells.

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It is known that carrier solvents used in commercial formulations of ES can mustxic effects of their own toward organisms, and they might contribute to the dysfunction of the endocrine system. To our knowledge, there are no published data reporting the possible effects of acetone exposure on GnRHs or GtHs cell populations in in vivo studies. In our experiments, exposure of *C. dimerus* larvae to acetone resulted in an increase of βFSH ir-cell nucleus/cytoplasm area ratio and also a decrease in mean cytoplasmic density, resulting in a possible increase in the release of FSH protein. Hallare et al. (2006) found that *D. rerio* embryos exposed to acetone showed induction of heat shock protein production, accelerated hatching, and decrease of embryo heart rate. Mac and Seelye (1981) found that the fries of *Salvelinus namaycush* exhibited higher growth rates compared with water controls. Other carrier vehicles also affect the HPG axis, as was reported by Harris et al. (2001), in which chronic exposure of *O. mykiss* to methanol led to alterations in pituitary content and secretion of FSH. In view of these precedents, acetone may have the potential of modulating the endocrine system in addition to causing other effects. Therefore, the use of acetone as a carrier vehicle in fish endocrine-disruption studies should be re-evaluated.

In conclusion, sublethal ES exposure affected some aspects of the neuroendocrine system of the hypothalamus-pituitary axis in *C. dimerus* larvae before gonadal sex differentiation. Morphometrical analysis indicated that ES affects GnRH I and βFSH levels after long-term exposure, but these responses seemed to be insufficient to affect gonadal differentiation at this stage of development. However, these changes could lead to a negative reproductive outcome by impairing sexual differentiation, maturation, and/or reproductive events, provided that the alterations in the neuroendocrine system continue with time. Several studies on pesticides have been performed exposing fish in a continuous manner; however, exposure to pesticides in the environment occurs mostly through agricultural runoff in pulses. Because multiple pesticide application events take place in a single season, the results obtained in this study could be extrapolated, with the proper cautions, to natural fish populations. This work also demonstrated that *C. dimerus* larvae is an interesting model organism in which many of the morphometric and ontogenetic basic features of their reproductive axis have already been investigated, thus providing a solid platform for further toxicologic and physiologic studies on the effect of several endocrine disrupters on neuroendocrine neurons/pituitary cells in early life stages of teleost fish. Summarizing, the present study is expected to be a tool for assessment of possible risks of ES exposure on fish larvae populations.

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