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Endosulfan affects GnRH cells in sexually differentiated juveniles of the perciform *Cichlasoma dimerus*

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ABSTRACT

Endosulfan (ES) is an organochlorine pesticide widely used in agriculture despite its high toxicity towards non-target organisms such as fish. It has been demonstrated that ES can cause negative effects on aquatic animals, including disruption of hormonal systems. However, the alterations produced by this pesticide on the reproductive axis of fish prior to sexual maturity, as well as possible modes of action have hardly been studied. This study aimed at assessing the effect of waterborne exposure to the pesticide ES on the reproductive axis during sexual differentiation of juveniles of the South American freshwater cichlid fish *Cichlasoma dimerus*. No mortality was observed due to ES subchronic exposure (90 days post-fertilization). Exposure to ES did not affect body weight nor morphometric parameters, indicating that larvae nutritional state was not affected. Timing of sexual differentiation, gonadal morphology and sex ratio were likewise not altered by ES. However, ES acted as an endocrine disrupting chemical in this species as the morphometry of gonadotropin-releasing hormones (GnRH) producing cells was altered. Exposure to ES altered nuclear area, cell area and nucleus/cytoplasm ratio of GnRH II neurons, and cell and nuclear area and diameter of GnRH III neurons. Interestingly, in our previous study, exposure before sex differentiation (30 day exposure) caused no alteration to GnRH II and III, and did alter GnRH I and FSH cells. These alterations could lead to changes in circulating hormone levels, especially when fish are exposed for prolonged periods, ultimately impairing reproductive fitness. *C. dimerus* juveniles can be an interesting biological model to perform toxicological studies with the intent to assess early disruption endpoints in the reproductive axis during development.

1. Introduction

Waterbodies act as reservoir of chemical compounds of domestic, industrial and agricultural use (Anderson et al., 1987; Boudou and Ribeyre, 1997), including organochlorine pesticides, considered to be hazardous since they are very persistent and ubiquitously found in the environment (Donohoe and Curtis, 1996; Palmer and Palmer, 1995). Due to their potential long term negative impact, their use in global agriculture has been largely restricted (RAP-AL, 2008; United Nations, 2009). One major exception in recent years has been endosulfan (ES; 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3benzo-dioxathiepin-3-oxide), cyclodiene pesticide vastly used for the control of insects and mites in crops of high commercial value (RAP-AL, 2008). Following its classification as a Persistent Organic Pollutant (POP) by the Stockholm Convention on POPs in 2010, ES use is in the process of being phased out worldwide (POPRC, 2010). However, this insecticide, introduced in Argentina in the 50s, can be detected in agricultural soils, sediments and surface waters of lakes and river basins, given its intense agricultural use over the last few years in rotational crops throughout the country (CASAFE, 2009), particularly on soy crops. Commercial formulations of ES consist of a mixture of two isomers, alpha and beta in a 70:30 ratio. ES alpha is more toxic than ES beta for fish and aquatic invertebrates. The mixture exhibits an intermediate toxicity (Wan et al., 2005). Environmental ES concentrations in Argentina range from 0.1 to 10.8 μg/kg dry weight in soils, and 0.38 μg/L ES α and 0.7 μg/L ES β in several river surface waters (Arias et al., 2010;

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Baudino et al., 2003; González et al., 2009; Jergentz et al., 2005; Lo Nostro et al., 2008; Miglioranza et al., 2004; Peluso et al., 2011); even though according to Argentinean laws, ES freshwater surface water concentrations must not exceed 0.02 μg/L for either isomer (Law No 24.051 under the Regulatory Decree No 831/93, 1993).

In fish, ES can act as neurotoxic producing irritability, aggressiveness, hyperactivity and seizures (Carlson et al., 1998; Salvo et al., 2008; Swarup et al., 1981), through neurodegeneration in different brain regions, necrosis and gliosis (Giusi et al., 2005; Sarma et al., 2010). In a previous study, we found that sexually undifferentiated larvae of the Perciform fish *Cichlasoma dimerus* exposed to 0.1 μg/L ES for 30 days showed altered gonadotropin releasing hormone I (GnRH I) and beta follicle-stimulating hormone (βFSH) brain/pituitary content as evidenced by lower and higher nucleus/cytoplasm area ratios respectively (Piazza et al., 2011). In vertebrates, brain GnRHs, regulate synthesis and release of gonadotropins (GtHs) from the pituitary (Cerdá-Reverter and Canosa, 2009; Okuzawa and Kobayashi, 1999). Of the three different forms of GnRH expressed in Perciformes (Pandolfi et al., 2005; White and Fernald, 1993), GnRH I exhibits a primary hypophysiotropic role, with abundant fibers present in the anterior pituitary, showing a strong correlation between GnRH I expression in the brain and gonadal activity (Senthilkumaran et al., 1999). In addition to other reported roles such as melatonin release from the pineal gland (Servili et al., 2010), fish GnRH II has also been shown to induce GtHs release (Chang et al., 2009), although no fibers extending to the pituitary have been evidenced in *C. dimerus* (Pandolfi et al., 2005). The third form, GnRH III is involved in regulating reproductive behavior in both males and females (Ogawa et al., 2006; Ramakrishnan and Wayne, 2009; Tubert et al., 2012).

Pituitary GtHs, FSH and luteinizing hormone (LH), play a critical role in the control and regulation of gonadal development, gametogenesis and gonadal steroidogenesis in teleosts (Devlin and Nagahama, 2002; Yaron et al., 2003). Reduced GtH neurosecretory activity can lead to delayed sex differentiation, and reduced fecundity; all reported effects of ES action under sub-lethal exposure (ranged from 0.01 to 1.4 μg/L) in fish (Balasubramani and Pandian, 2008; Gormley and Teather, 2003; Shukla and Pandey, 1986). Alterations in germ cell distribution and deviation to female sex proportion have also been reported as sub-lethal effects of the pesticide on larvae (Teather et al., 2005; Willey and Krone, 2001). However, on our previous study on sexually undifferentiated larvae, alterations caused by ES at the hypothalamus and pituitary did not affect the onset of gonadal differentiation at the studied stage of development, 30 days post fertilization (dpf) (Piazza et al., 2011).

Therefore, the aim of the present research study was to examine if a three month sub-lethal exposure to ES, can also affect morphometric parameters and anatomical localization of GnRHs and/or GtHs cell populations, as well as sex proportion, i.e. sexually differentiated juveniles at 90 dpf, in *C. dimerus* (Teleostei, Perciformes), a cichlid fish relevant to South American riverine ecosystems, whose sex differentiation and time of appearance of GnRH and GtHs expressing cells and fibers have been already studied in normal conditions in laboratory reared larvae (Meijide et al., 2005; Pandolfi et al. 2002, 2005).

2. Materials and methods

2.1. Animals

Adult *C. dimerus* were captured in two sites on Esteros del Riachuelo, Corrientes, Argentina (27°35′S, 58°45′O). After transfer to laboratory facilities, animals were held in 200 L aquaria with a layer of gravel on the bottom, in filtered tap water at 26 ± 1 °C, pH 7.3, and a 14 h:10 h photoperiod. Fish were allowed to acclimate for two weeks and were fed daily with a pelleted commercial diet (Tetra® food sticks, Germany). Newly hatched larvae (2 days post-fertilization (dpf)) of 2 ± 0.5 mm (total length) were collected from fresh spawns obtained from breeding couples established within the aquaria. When larvae started to swim (8 dpf), they were fed with Artemia sp. *nauplii* twice a day during the first 20 days and with ground flake food thereafter. Guidelines on the care and use of fish in research and testing from the Canadian Council on Animal Care (CCAC, 2005) and local regulations of our faculty were followed.

2.2. Sublethal chronic assays

Exposure concentrations of ES (94.99% purity, using a stereoisomer mixture of α:β 70:30), of 0.03 and 0.1 μg/L were selected based on the previously obtained 96-h acute toxicity test (0.3 μg/L for larvae at 10 dpf; Piazza et al., 2011) and prepared from a 0.01 mg ES/mL acetone stock solution added to filtered tap water (final solvent concentration of 0.001%). New stock solution was prepared before every media renewal. Actual ES concentrations were measured in water samples 15 min after renewal by gas chromatograph-electron capture detector (US Environmental Protection Agency SW846 M8081A, 1996), resulting in 0.02 (for nominal 0.03) and 0.08 (for nominal 0.1) μg/L.

Although no ES was detected in different streams of the mesopotamic region (including Corrientes province), in order to avoid a possible maternal transfer of contaminants or epigenetic effect, all larvae clutches were distributed between all experimental conditions – control, solvent and ES – so that if present this factors would be equal between all treatments to control litter effect. Then, larvae of 2 dpf were exposed to ES by way of immersion under semistatic conditions. Each concentration was tested four times with 10 individuals per test group in 2.5 L glass tanks. Control treatments (with and without vehicle) were also performed in duplicate. Acetone was added to the control vehicle test group in an amount equal to that present in the highest concentration of ES employed. Media was renewed every 48 h, taking into account ES degradation (60% remaining at 50 h) and mortality of larvae caused by stress of manipulation.

Larvae mortality and gross anatomic abnormalities (lordosis, scoliosis, body swelling, depigmentation) were observed and recorded throughout the experiment.

After the 88-day exposure period (90 dpf), larvae were weighed, their standard length was measured, and they were killed by decapitation under anesthesia (Fish Calmer®, Jungle Lab., USA). Condition factor (K=(Weight/Length^3)*100) was also calculated.

2.3. Tissue fixation

Following Piazza et al. (2011), head and trunks were fixed in Bouin's solution for 24 h at 4 °C, then dehydrated and embedded in Paraplast® (Fisherbrand, USA). Heads were transversally sectioned at 10 μm for immunohistochemical techniques, and trunks were transversally sectioned at 7 μm and stained with hematoxilin-eosin for sex determination.

2.4. Single-label immunohistochemistry

Sections were deparaffinized in xylene, rehydrated through a series of graded ethanols to phosphate-buffered saline (0.05 M PBS, 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% non-fat dry milk at RT,

incubated in a closed moist chamber with their specific primary antiserum overnight at 4 °C – anti-sbGAP for GnRH I, anti-cIIGAP for GnRH II and anti-sGAP for GnRH III 1:600 (*Dicentrarchus labrax*, donated by Dr. J. A. Muñóz Cueto, Universidad de Cádiz, Spain); anti-βFSH 1:1000, anti- βLH 1:2000 (*Fundulus heteroclitus*, donated by Dr. A. Shimizu, NRIFS, Fisheries Research Agency, Japan), washed in PBS, and finally incubated for 45 min with their complementary secondary biotinylated antibody (Vector, USA) at RT. Amplification of the signal for GnRH detection was carried out using a tyramide based-signal amplification kit (CSA-Peroxidase kit, Dako, USA) following the manufacturer's instructions. For βFSH and βLH, amplification of the signal was achieved by incubation with peroxidase-conjugated streptavidin (Dako, USA) diluted 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 5400, Nikon). Settings (light intensity, opening of the condenser, etc.) of the microscope and the camera were maintained constant.

In order to ensure the correct topographic localization of the different GnRH cell types and their projections, brain atlases of *Dicentrarchus labrax* (Cerdá-Reverter et al., 2001a, 2001b) and *Haplochromis (Astatotilapia) burtoni* (Fernald and Shelton, 1985) were used as guidance, as well as the previously described brain anatomy, and GnRH, βFSH and βLH immunoreactive (ir-) cells localization in adults of *C. dimerus* by Pandolfi et al. (2005, 2006). Also, as stained cells are located within confined brain regions, all slides with ir-cells were considered for measurements.

2.5. Antisera

The specificity of antisera in *C. dimerus* has already been previously established by pre-adsorption tests with their respective antigens and by Western blot analysis in our lab (see Piazza et al., 2011). To avoid false positives some slices were incubated with PBS instead of either the primary or secondary antibody.

2.6. Epitope unmasking

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

2.7. Morphometrical analysis of GnRH, βFSH and βLH producing cells

Nuclear and cytoplasmic two-dimensional area (μm^2) and mean diameter (μm), as well as optical density of the immunostain were measured in ir-cells. Given that, at this stage of development it is difficult to find isolated cells with clearly discernible cytoplasm and nuclear perimeters in the plane of the section for each fish, since cells mostly form clusters, on average 10 random ir-cells were considered. All parameters were analyzed using an image processing program (Image pro-plus® 4.5 software, Media Cybernetics). All cells were measured in the same brain regions for all fish. For optical density, background density was subtracted from each value to reduce variability in the results. The parameters considered in this study have already been satisfactorily used in other studies for this and other species (Parhar et al., 2001; Shimizu et al., 2008; Piazza et al., 2011). Morphometric parameters were considered substitute indicators of cellular activity (Costa and Paula, 2006; Filippa and Mohamed, 2010; Morandini et al., 2014) as size of larvae precludes measuring plasma levels.

2.8. Statistical analysis

Mortality, body weight and size results were statistically analyzed using factorial analysis of variance (ANOVA). Morphometrical parameters were analyzed with a two way nested ANOVA design. When data did not meet the ANOVA assumptions (homogeneity of variance, normality), they were log transformed prior to analysis. Non-parametric analysis (Kruskal–Wallis test) was applied when data could not be assumed to be normally distributed. When significant differences were found Tukey's post-hoc analysis (parametric) or Dunn's test (non-parametric) were performed (STATISTICA 8.0; StatSoft, Inc.). To analyze sex ratio, a Chi square test was performed to a 50:50 proportion. Values were considered significantly different if $p < 0.05$. In all instances data are presented as mean ± S.E.M.

3. Results

3.1. Mortality

Larvae survival (mortality) was not affected by ES exposure, however those animals exposed to either ES concentration or to acetone showed a tendency towards higher mortality values than control fish (ANOVA, $p=0.22$; Table 1).

3.2. Body size and weight

No external anatomical abnormalities were observed at the end of the experiment. Weight, standard length and condition factor did not differ between control and exposed larvae at the end of the experiments (ANOVA, $p=0.3$; Table 1). No significant differences were found between sexes for any of the aforementioned parameters (ANOVA, $p=0.9$).

3.3. Sex ratio

Sex of 90 dpf juveniles was determined under a stereoscopic microscope. Ovaries and testes were easily distinguishable from each other and showed no macroscopic or microscopic alterations (not shown). Sex was confirmed by histology. Sex ratio did not differ from 50:50 (female:male) between treatments being 51:49 for control, 45:55 for vehicle, 46:54 for 0.03 μg/L and 45:55 for

Table 1
Larvae mortality (%), total body weight (mg), standard length (mm) and condition factor (mg/mm^3) at the end of the experiment in the different treatments. No statistical differences were observed between any of the parameters (ANOVA, $p > 0.5$).

Treatment	Mortality (%)	Total body weight ± SEM (mg)	Standard length ± SEM (mm)	Condition factor ± SEM (mg/mm^3)
Control	50	77.8 ± 6.4	12 ± 0.4	4.2 ± 0.9
Vehicle	66	78.1 ± 5.2	12 ± 0.2	4.7 ± 1.3
0.03 μg/L	70	76.2 ± 5.4	11.4 ± 0.4	4.3 ± 0.6
0.1 μg/L	68	71.3 ± 4.4	11.8 ± 0.4	4.1 ± 1.4

Table 2
Values of cellular area (CA), mean cellular diameter (MCD), nuclear area (NA) and mean nuclear diameter (MND) of the different cell types in 90 dpf juveniles from control treatments. Numbers indicate mean ± SEM. * Significant differences between sexes (2 way nested ANOVA 2F, $p < 0.05$; Tukey test, $p < 0.0003$).

Cell type dpf	CA 90	(μm^2) 90	MCD 90	(μm) 90	NA 90	(μm^2) 90	MND 90	(μm) 90
Sex	♀	♂	♀	♂	♀	♂	♀	♂
GnRH I	20 ± 2	18 ± 1.5	5 ± 0.2	5 ± 0.2	5 ± 0.5	4 ± 0.3	3 ± 0.2	2 ± 0.1
GnRH II	258 ± 16	284 ± 20	18 ± 0.6	20 ± 1	110 ± 6	138 ± 9	12 ± 0.4	14 ± 1
GnRH III	254 ± 19	153 ± 8*	18 ± 0.9	13 ± 0.4*	86 ± 6	48 ± 4*	10 ± 0.4	8 ± 0.3*
βFSH	41 ± 3	44 ± 2	7 ± 0.3	8 ± 0.2	18 ± 1	18 ± 1	4 ± 0.2	5 ± 0.2
βLH	66 ± 4	69 ± 3	9 ± 0.3	9 ± 0.3	27 ± 2	29 ± 3	6 ± 0.2	6 ± 0.3

0.1 μg/L (Chi-Square test, $p > 0.05$).

3.4. Morphometrical analysis

3.4.1. GnRH I ir-neurons (salmon GnRH)

GnRH I ir-neurons were scattered along the ventral portion of the telencephalon through the pre-optic area (POA). These neurons were located separate from each other and were the smallest within the group of GnRH producing neurons (mean cellular area from control larvae of 20 ± 2 μm^2 and 18 ± 1.5 μm^2 for females and males respectively; Table 2; Fig. 1A–c). There was a significant difference between sexes for all parameter analyzed. Even though, ES and acetone differed from control animals for most parameters evaluated (2 way nested ANOVA, $p < 0.05$; Tukey test $p < 0.05$) no differences were observed between ES treatments and acetone (Fig. 2).

3.5. GnRH II Ir-neurons (chicken II GnRH)

GnRH II ir-neurons were big sized cells located in the midbrain *tegmentum* (mean cell area: 258 ± 16 μm^2 and 284 ± 20 μm^2, females and males respectively, Table 2). These cells were frequently found in pairs, near to the ventricular ependyma and to blood vessels usually located in that region (Fig. 1D–F). Three month exposure to 0.1 μg/L ES caused lower nuclear area than acetone exposure (2 way nested ANOVA, $p < 0.03$; Tukey test, $p = 0.01$) (Fig. 2B). Mean nuclear diameter showed the same pattern, though no significant differences were observed (Fig. 2E). Cell area was increased upon exposure to 0.03 μg/L ES (Fig. 2A). Nucleus/cytoplasm area ratio was smaller in animals exposed to either ES concentration with respect to vehicle (Fig. 2C). No differences were observed for optical density (Fig. 2F). As no difference was observed between sexes, data were plotted together for all parameters.

3.6. GnRH III Ir-neurons (seabream GnRH)

GnRH III ir-neurons were found in the ventral forebrain on the caudal olfactory bulb, mostly clustered together (Fig. 1G–I). These neurons had a mean cell area of 254 ± 19 μm^2 and 153 ± 8 μm^2 for females and males respectively, showing significant differences between sexes (2 way nested ANOVA, $p < 0.05$; Tukey test, $p < 0.0003$, Table 2). Animals exposed to 0.03 μg/L ES showed the highest cell and nuclear area and diameter (2 way nested ANOVA, $p < 0.008$; Tukey test, $p < 0.0008$), while those exposed to 0.1 μg/L showed the lowest values in the same parameters (2 way nested ANOVA, $p < 0.008$; Tukey test, $p < 0.05$) (Fig. 2A, B, D, E). No differences were observed for the remaining parameters (Fig. 2C, F) or between sexes (data plotted together).

3.7. βFSH ir-CELls

βFSH ir-cells were found forming clusters in the proximal *pars distalis* (PPD) and along the external border of the *pars intermedia* (PI) of the pituitary (mean cell area of 41 ± 3 μm^2 and 44 ± 2 μm^2, for females and males respectively; Fig. 3A–C; Table 2). As there was a significant difference between sexes for nuclear area and mean nuclear diameter, they were plotted by sex. Though not statistically different from vehicle, juveniles at 90 dpf exposed to 0.1 μg/L ES showed a higher cell area than those exposed to 0.03 μg/L ES (2 way nested ANOVA, $p < 0.006$; Fig. 4A). No differences were observed for any of the remaining parameters (Fig. 4B–F).

3.8. βLH ir-cells

Cells showing immunoreactivity for βLH were located in the ventral, central and marginal regions of the PPD in the pituitary (mean cell area of 66 ± 4 μm^2 for females and 69 ± 3 μm^2 for males; Fig. 3D–F; Table 2). No differences were observed between experimental conditions for cell area and mean diameter, nuclear area and mean diameter, or nucleus/cytoplasm area ratio (Fig. 4A–E). Mean cytoplasmic optical density in cells of fish exposed to 0.03 μg/L ES increased when compared to 0.1 μg/L ES (2 way nested ANOVA, $p < 0.02$; Tukey test, $p < 0.002$; Fig. 4F). No differences between sexes were observed for any other parameter (data plotted together).

4. Discussion

Response to pollutants exposure in fish depends on the life cycle, habitat, food, biology of each species, among other factors. Embryos and larvae are the most sensitive stages within the teleost life cycle. A high natural mortality before reaching the juvenile stage – around 90% – due to high predation and/or food shortage is usual in most egg spawning species. Mortality rates diminish as larvae grow; however, when exposed to contaminants, motility and sensory abilities can be altered resulting in increased mortality and serious consequences at the population level (Alvarez, 2005; von Westernhagen, 1988). Neither ES nor the solvent acetone significantly altered mortality of *C. dimerus* larvae, either at 30 dpf (Piazza et al., 2011) or 90 dpf (present study) at the same concentrations.

Non-water soluble chemicals are dependent on vehicle solvents in order to allow dissolution. Lipophilic compounds such as ES require organic solvents to enable their dispersion in water, so commercial formulations use a mixture of emulsifiers, carriers and additives. Coformulants improve the agricultural usability of pesticides, allowing better dispersion, distribution, penetration and persistence on crops. However, they may have toxic effects of their own on non-target animals, either through direct or indirect, specific or non-specific mechanisms (Hutchinson et al., 2006; Maes et al., 2012). In the case of acetone, it is capable of inducing heat shock protein production in *Danio rerio* embryos and thus accelerates hatching and decreases heart rate (Hallare et al., 2006).

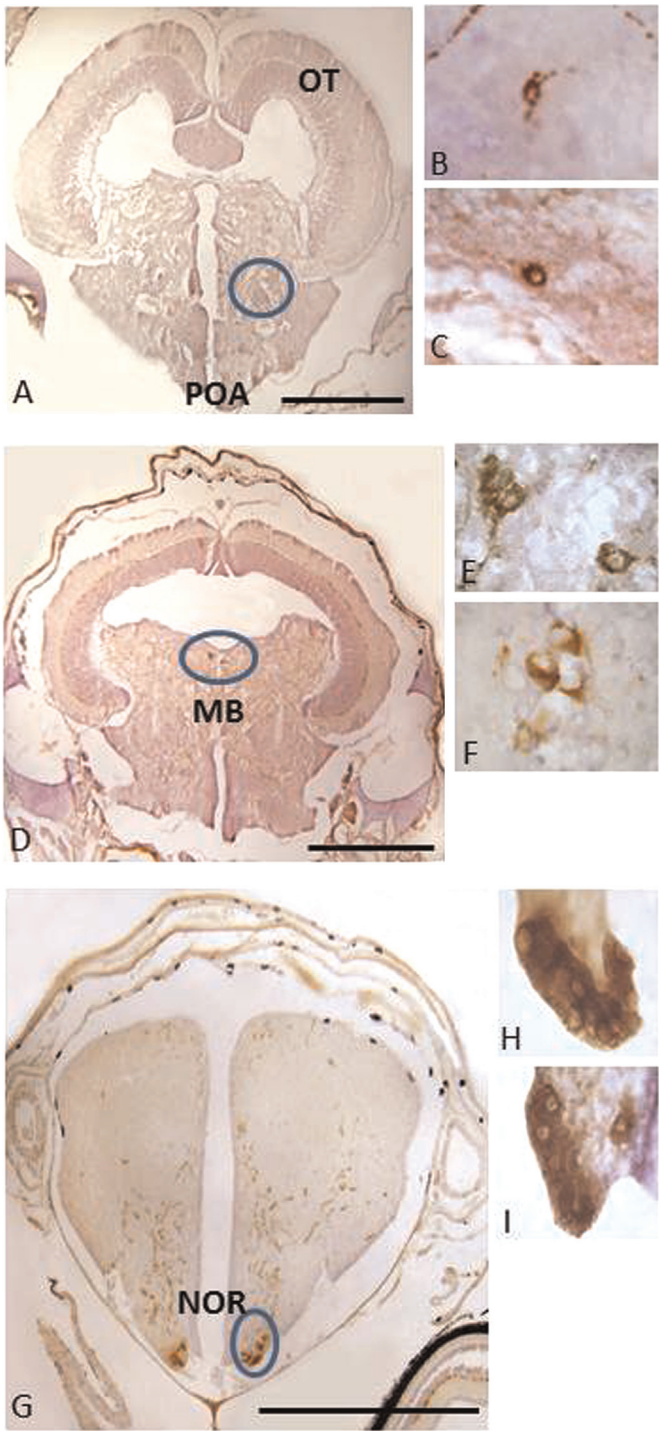

Fig. 1. Topographic light-microscope photograph of different brain cross sections of *Cichlasoma dimerus* 90 dpf juveniles, (A) brain preoptic area (POA), (D) midbrain *tegmentum* (MB), (G) *nucleus olfacto retinalis* (NOR). Neuron detail from females and males respectively: (B), (C) GnRH I ir-neurons. (E), (F) GnRH II ir-neurons, (H), (I) GnRH III ir-neurons. ot: optic tectum. Scale bares: (A), (D) and (G): 500 μm, (B), (C), (D), (E), (H) and (I): 1000X.

Larvae of *Salvelinus namaycush* exposed to this solvent, exhibited higher growth rates than fish kept in compound-free water (Mac and Seelye, 1981). In our previous study, acetone affected FSH and GnRH I cell populations, however exposure did not affect other brain cell populations, larvae survival, or growth (Piazza et al., 2011). Acetone was once again used in this study so that data from the previous study (exposure for 30 days) and this one (exposure for 90 days) could be readily compared. Additionally, as the use of a solvent is necessary for the preparation of ES solutions, acetone was chosen as it showed lower toxicity to early developmental stages than other solvents (Maes et al., 2012). An effect of acetone exposure to GnRH I-producing neurons was observed in this study, which could mask the effects of ES. Its use for endocrine disruptive studies should consider this action.

Gormley and Teather (2003) observed reduced length in *Oryzias latipes* larvae exposed to ES for one week when larvae were exposed before hatching; however, after a 4-week exposure or when larvae where exposed after hatching no difference in length was found between control and exposed fish. Beyger (2009) did not found altered growth for ES-exposed *Jordanella floridae* larvae, in agreement with our results for *C. dimerus* larvae, where growth was unaffected by ES. Furthermore, *K* values of exposed larvae reflected a good nutritional status.

Successful reproduction depends on individuals having gone through normal gonadogenesis, a complex process involving cellular and histological differentiation that starts during early ontogeny. Environmental toxicants may affect the endocrine control of gonad development in larvae. Embryos of *D. rerio* exposed to low concentrations of ES showed altered primordial germ cells distribution along the antero-posterior axis (Willey and Krone, 2001). Reduction in the number of spermatogonia and spermatids, loss of Sertoli cells and thickening of the seminiferous tubules in males of the same species, resulting in delayed sexual maturity and fertilizing capacity, was also reported following ES exposure (Balasubramani and Pandian, 2008). In *C. dimerus*, Da Cuña et al. (2011, 2013) reported that adult males exposed to subchronic environmentally relevant concentrations of ES showed testis disorders. For early life stages, results of the present study showed that, under the experimental conditions tested, ES did not cause morphological and/or histological gonadal alterations in juveniles.

Several authors have reported that ES has an estrogenic effect in vitro, although it has low affinity for the estrogen receptor (Gale et al., 2004; Petit et al., 1997; Wozniak et al., 2005). It has also been described as an androgen antagonist and a weak inhibitor of aromatase in mammalian cells (Andersen et al., 2002). In *D. rerio* exposed to low concentrations of ES in a pulsatile fashion, sex ratio was skewed towards males (Balasubramani and Pandian, 2008), whereas higher concentrations shifted sex proportion towards females and undifferentiated juveniles. In contrast to these reports, the gonads of ES-exposed *C. dimerus* differentiated normally into females or males, without an imbalance in sex ratios, as described for this species by Meijide et al. (2005), suggesting that ES does not alter gonadal morphology or sex ratio at the concentrations and exposure period tested in this species. It would prove of interest to study if exposure of newly fertilized eggs and/or use of commercial formulations rather than the active ingredient alone, could result in a different outcome regarding sex ratio.

Chemicals substances that are capable of mimicking sex steroids can also alter the hypothalamic–pituitary–gonadal axis of vertebrates, since these hormones regulate gonadotropin synthesis and secretion through feedback mechanisms. Subchronic exposure of *Oreochromis mossambicus* to ES caused gonadotrophs and thyrotrophs with vacuolated or granular cytoplasm, cell enlargement and, in some cases, nuclear damage (Shukla and Pandey, 1986). On the contrary, and similarly to our previous results in 30-day old larvae (Piazza et al., 2011), 90-day exposure to ES did not cause *C. dimerus* brain tissue damage, nor any anomaly in the anatomic localization or time of appearance of GtHs or GnRHs cell populations.

In contrast to our previous study where FSH cells showed an increased in size when larvae were exposed for 30 days to 0.1 μg/L ES (Piazza et al., 2011), limited sensitivity of FSH cells was found upon 90 days ES exposure in this study. Pesticide exposure did not alter any of the morphometric parameters measured, save for a

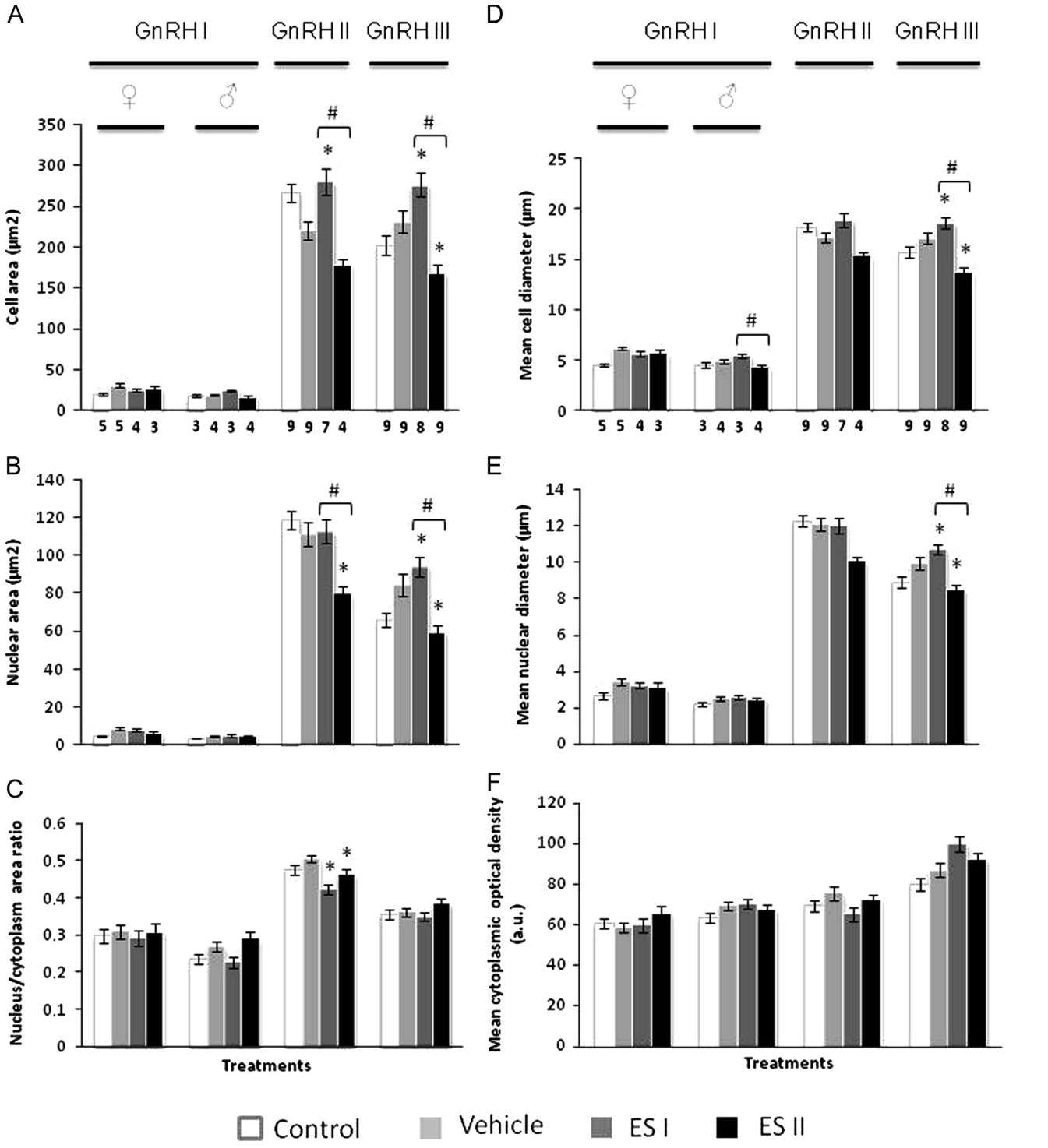

Fig. 2. (A) cellular area, (B) nuclear area, (C) nucleus/cytoplasm area ratio, (D) mean cellular diameter, (E) mean nuclear diameter, (F) mean cytoplasmic optical density of GnRH I, II, III ir-neurons of 90 dpf juveniles. When no significant differences were found between males and females, data for both sexes was plotted together. Bars show mean ± SEM. Numbers indicate sample size for each group. (*) Significant difference between treatments and vehicle. (#) Significant difference between treatments. Values were considered significantly different when $p < 0.05$ (2 way nested ANOVA, Tukey's test).

difference in cell area between both ES concentrations tested. Similarly, morphometric parameters of LH-producing cells were not affected by pesticide exposure, except for a slight increase in optical density, possibly due to hormone accumulation. LH plays an important role in gonadal maturation in fish (Zohar et al., 2010), so long-term exposure to ES during this period could diminish the reproductive fitness. Even though results indicate that gonadotrophs are not sensitive to the presence of the pesticide after prolonged exposure, confounding effects due to the solvent cannot be excluded.

Regarding GnRH, in our previous study, *C. dimerus* larvae exposed for 30 days to 0.1 µg/L of ES showed that GnRH I ir-neurons decreased their nucleus/cytoplasm ratio, attributed to a reduced nuclear size, suggesting diminished cell activity (Piazza et al., 2011). Ibrahim et al. (1986) observed that cell size correlated with hormone serum levels suggesting increased hormone synthesis

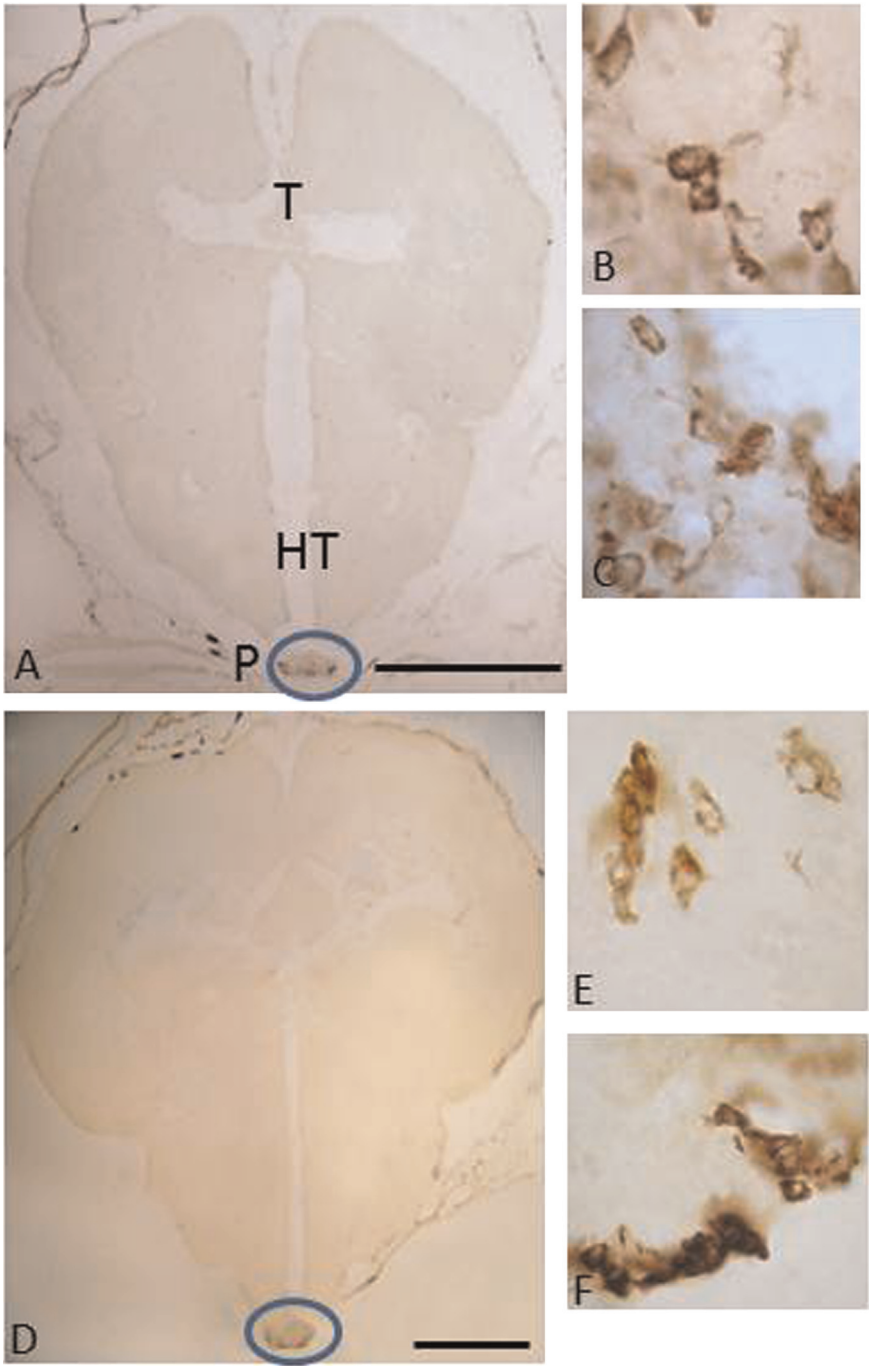

Fig. 3. Topographic light-microscope photograph of different brain cross sections at pituitay level of 90 dpf juveniles. (A) Pituitary rostral pars distalis and pars intermedia showing βFSH ir-cell location. (D) Pituitary proximal pars distalis showing βLH ir-cell location. Cell detail of female and male respectively: (B) and (C) βFSH ir-cells. (E) and (F) βLH ir-cell. HT: hypothalamus, P: pituitary; T: telencephalon. Scale bars: (A): 500 μm, (D) 250 μm, (B), (C), (E) and (F): 1000X.

and/or secretion. Also, in *C. dimerus*, nuclear area of steroidogenic adrenal cells positively correlated with cortisol plasma levels (Morandini et al., 2014).

Previous studies in adults of this species state that innervation of the pituitary by GnRH I is associated with increased plasma concentration of FSH at the onset of ovarian differentiation (42 dpf) (Pandolfi et al., 2002). Therefore, the effects on morphometry of FSH cells after 30-day exposure to ES would reflect a direct effect of the pesticide on gonadotrophs rather than an effect mediated by GnRH (Piazza et al., 2011). After 90-day exposure both direct and indirect effects of ES could affect FSH-producing cells, as innervations are well established at this later stage; however no effects were evident neither on GnRH I neurons nor FSH cells.

While GnRH I acts as a gonadotropin releasing factor, the wide distribution and large number of neurons corresponding to GnRH II and III in fish brain indicate that these neuropeptides serve important functions, many still unclear (Lethimonier et al., 2004). Immunoreactive fibers and receptors for these two neuronal populations are close to brain sensory areas, implying a modulatory role for both neuropeptides (Chen and Fernald, 2006; Forlano et al., 2000; Kawai et al., 2009; Maruska and Fernald, 2010; Millar, 2003; Soga et al., 2005). GnRH II has been linked to modulation of sexual stimuli, communication between individuals and/or modulation of pineal function and melatonin secretion (Maruska and

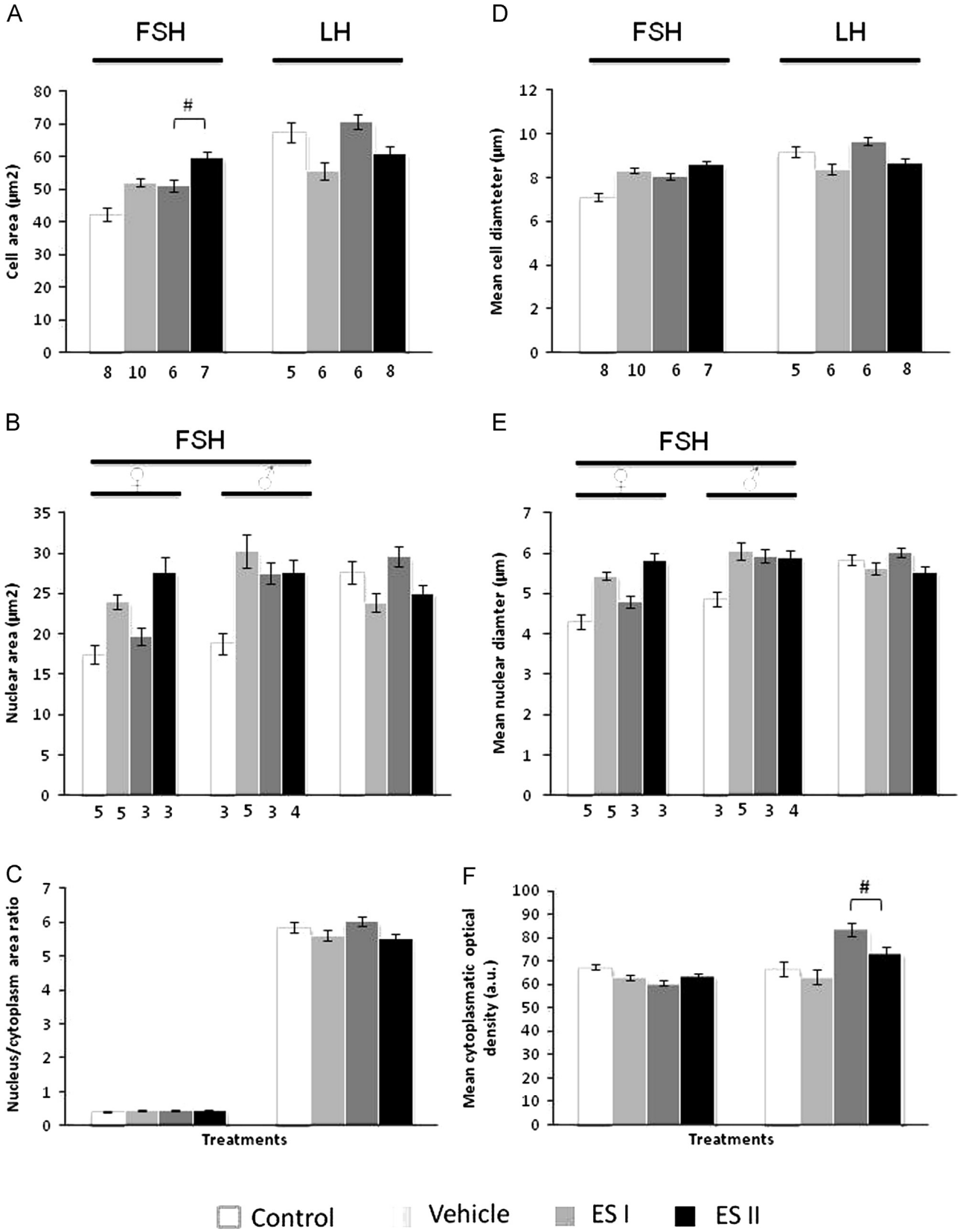

Fig. 4. (A) Cellular area, (B) nuclear area, (C) nucleus/cytoplasm area ratio, (D) mean cellular diameter, (E) mean nuclear diameter, (F) mean cytoplasmic optical density of βFSH and βLH ir-cells in 90 dpf juveniles. When no significant differences were found between males and females, data for both sexes was plotted together. Bars show mean ± SEM. Numbers indicate sample size for each group. (*) significant difference between treatments and vehicle. (#) significant difference between treatments. Values were considered significantly different when $p < 0.05$ (2 way nested ANOVA, Tukey's test).

Tricas, 2007; Servili et al., 2010), while GnRH III appears to regulate reproductive behavior in adults (Keller-Costa et al., 2015; Tubert et al., 2012; Uchida et al., 2005).

In our previous study, GnRH II and III were insensitive to the pesticide after 30 days of exposure, since ES did not affect morphometric parameters or optical density of either neuronal type (Piazza et al., 2011). In contrast, following a 3-month exposure period, both cell types were affected by ES. Impaired neuronal growth could result in reduced neuropeptide synthesis and therefore altered social behavior of individuals through modulation of sensory systems – visual, auditory and olfactory (Canosa et al., 2008; Servili et al., 2010), known functions of GnRH systems in fish (Eisthen et al., 2000; Maruska and Tricas, 2011; Stell et al., 1987). Given the neuroendocrine role in the reproductive cycle of both hormones, through decreased activity of GnRH II, as suggested by the decreased nucleus/cytoplasm ratio for both ES concentrations, and GnRH III, as seen by the lower nuclear and cellular area with the highest ES concentration tested, sexual behavior such as mate choice, courtship, nest building and number of eggs laid could be affected, with detrimental effects on the number of individuals in future generations. These results indicate that GnRH II and III neurons are sensitive to the presence of ES when exposed for a prolonged period of time. Interestingly, GnRH I and FSH were the cell types affected when exposure was shorter, ending before sexual differentiation.

Taken together both studies, it is observed that cell types can respond differently depending on the exposure time. Alteration of the reproductive axis in larvae via these effects can lead to impaired sexual development and/or future reproductive success.

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