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Mechanism of action of endosulfan as disruptor of gonadal steroidogenesis in the cichlid fish *Cichlasoma dimerus*

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ABSTRACT

The organochlorine pesticide endosulfan (ES) is used in several countries as a wide spectrum insecticide on crops with high commercial value. Due to its high toxicity to non-target animals, its persistence in the environment and its ability to act as an endocrine disrupting compound in fish, ES use is currently banned or restricted in many other countries. Previous studies on the cichlid fish *Cichlasoma dimerus* have shown that waterborne exposure to ES can lead to both decreased pituitary FSH content and histological alterations of testes. As gonadotropin-stimulated sex steroids release from gonads was inhibited by ES *in vitro*, the aim of the present study was to elucidate possible mechanisms of disruption of ES on gonadal steroidogenesis in *C. dimerus*, as well as compare the action of the active ingredient (AI) with that of currently used commercial formulations (CF). Testis and ovary fragments were incubated with ES (AI or CF) and/or steroidogenesis activators or precursors. Testosterone and estradiol levels were measured in the incubation media. By itself, ES did not affect hormone levels. Co-incubation with LH and the adenylate cyclase activator forskolin caused a decrease of the stimulated sex steroids release. When co-incubated with precursors dehydroandrostenedione and 17αhydroxyprogesterone, ES did not affect the increase caused by their addition alone. No differences were observed between the AI and CFs, suggesting that the effect on steroidogenesis disruption is mainly caused by the AI. Results indicate that action of ES takes place downstream of LH-receptor activation and upstream of the studied steroidogenic enzymes.

1. Introduction

One of the main factors endangering aquatic wildlife is water pollution by anthropogenic contaminants, particularly pesticides (Wilcove et al., 1998). Their use in agriculture is therefore under constant monitoring to minimize risks to non-target species. Due to its persistence and potential biomagnification, endosulfan (ES; IOUPAC ID: 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) is currently recognized as one of the most hazardous organochlorine pesticides, having been included in the list of Persistent Organic Chemicals by the Stockholm Convention (United Nations C.N.703.2011.TREATIES-8). Though in the process of being phased out worldwide, ES is still widely used in several countries as a broad spectrum insecticide for commercial crops, with India and Israel as the largest producers (Rand et al., 2010).

Endosulfan is able to reach water bodies through runoff, with reported surface water levels range from 0.01 to 2.5 μg/L (Dalvie et al., 2003; Leong et al., 2007; Ballesteros et al., 2014); since the pesticide can bioaccumulate in tissues (bioconcentration factors in fish of 620–1344, Schimmel et al., 1977) organ levels in the range of 10–400 ng/g have been found in fish (Akhtar et al., 2014; Ballesteros et al., 2014; Menone et al., 2000; Polder et al., 2014; Singh et al., 2008). Once in contact with aquatic biota ES can interfere with the normal regulation of numerous biological processes, including fish reproduction (Han et al., 2011; Balasubramani and Pandian, 2014). In this regard, ES can act as an endocrine disrupting chemical (EDC), affecting hormonal regulation. In mammals, male rats orally administered with ES showed decreased plasma levels of TSH, increased levels of growth hormone and down-regulation of luteinizing hormone (LH) mRNA but elevated LH secretion following ES exposure (Caride et al., 2010). Decreased sperm number and motility, lower circulating testosterone levels, reduced expression of androgen receptor mRNA in testes and increased plasma LH have also been reported in male mice by effect of ES (Wang et al., 2014). In fish, exposure to ES of mature zebrafish (*Danio rerio*) resulted in

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decreased hatching rate, together with pathological alterations in testes and increased vitellogenin levels in males (Han et al., 2011). In juvenile catfish (*Clarias batrachus*), this pesticide caused a decrease of gonadotropin-releasing hormone mRNA levels in brain, together with increased ovarian aromatase activity (Chakrabarty et al., 2012). In the same species, ES decreased the expression of steroidogenic enzymes in testes at concentrations below those found in the environment (Rajakumar et al., 2012). *In vitro* assays showed that ES was able to decrease cortisol secretion upon stimulation with adrenocorticotropic hormone (ACTH) in adrenocortical and head kidney cells from rainbow trout (*Oncorhynchus mykiss*) (Dorval et al., 2003; Hontela et al., 2008).

Previous studies with the South American freshwater cichlid fish *Cichlasoma dimerus* (Kullander, 1983) showed that waterborne exposure to ES was capable of decreasing βFSH pituitary content and altering testes tissue structure in adults (Da Cuña et al., 2011, 2013), as well as altering GnRH and FSH producing cells in larvae and juveniles (Piazza et al., 2011, 2015). This freshwater cichlid fish is representative of Argentinian riverine ecosystems, and is recommended by local regulations as one of the suitable fish species for use in ecotoxicological testing (IRAM, 2008).

Several EDCs have been shown to act as either agonists or antagonists of steroid receptors *in vitro*, particularly the estrogen receptor, albeit with much lower affinities than endogenous hormones (Sanderson, 2006). In addition to receptor interaction, key enzymes involved in steroid hormone synthesis can also be important targets for disruption (Quignot et al., 2012). Most studies regarding the effects of pesticides on non-target species focus on either the active ingredient or a commercial formulation without analyzing the possible differences between them. Coadjuvants can contribute to the overall toxicity of the formulation and may not have the same effects caused by the active ingredient. Both antagonist and synergistic effects between the active ingredient and other components present in commercial formulations have been reported in terms of both genotoxicity and reproductive toxicity (Guilherme et al., 2012, 2014; Chaufan et al., 2014; Urióstegui-Acosta et al., 2014).

Based on our previous study indicating an *in vitro* inhibition caused by pure ES on the stimulatory effect of LH on steroidogenesis (Da Cuña et al., 2013), the aim of the present study was to identify some possible mechanisms of disruption exerted by ES, as well as to compare the mechanism of action of the active ingredient with that of two currently used commercial formulations of ES (Master® and Zebra Ciagro®) on the gonadal steroidogenesis of *C. dimerus*.

2. Materials and methods

2.1. Animals

Adult *C. dimerus* were captured at Esteros del Riachuelo, Corrientes, Argentina (27°35′S 58°45′W) and transported to the laboratory, where they were allowed to acclimate for a month in 100 L well aerated aquaria (dissolved oxygen 8 mg/L; 98.6% saturation) provided with external filtration, a bottom layer of gravel and filtered tap water (pH 7.3, hardness 55 mg/L). A temperature of 25 ± 1 °C and a photoperiod of 14:10 (L:D) were maintained throughout. Fish were fed daily with 90 mg commercial pellet food (Tetra food® sticks). Handling of animals was performed in compliance with local and international guides on animal welfare (National Research Council, 2011).

2.2. Chemicals and hormones

Endosulfan active ingredient (AI; α:β isomer ratio 70:30; chromatographic purity 95%) was donated by SENASA (Buenos Aires, Argentina). Commercial formulations of ES used (35% AI), Zebra Ciagro® (Ciagro SA, Argentina; CF1) and Master® (Cheminova, Argentina; CF2), were purchased from local suppliers. LH purified from pituitary of *Fundulus heteroclitus* specimens (Shimizu and Yamashita, 2002) was kindly provided by Dr. Shimizu (Fisheries Research Agency, Japan). Forskolin (FK), dehydroepiandrosterone (DHEA) and 17αhydroxyprogesterone ($17\alpha P_4$) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Gonad cultures

Adult fish were anesthetized (Fish Calmer; TX, USA), weighed (mean weight ± SD: 40.1 ± 3.1 g and 29.2 ± 7.7 g for males and females respectively) and killed by decapitation. Gonads were quickly dissected, removing proximal and distal ends. Both testes were sectioned into 5 small pieces (5–6 mg) and incubated in 150 μL Krebs-Ringer-HEPES-glucose medium (KRHG; 78 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_4PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 11.5 mM glucose, pH 7.4). Ovary fragments (45–50 mg) were incubated in 200 μL KRHG. Initially, all explants were mechanically disaggregated using fine needles and then washed in KRHG for 20 min at the same volumes indicated above. Culture medium was then replaced and supplemented with ES and/or the corresponding stimulatory or inhibitory factor according to each experiment (as detailed below). Dimethylsulfoxide (DMSO), used as solvent for ES, forskolin and the steroid precursors, was added in equal amounts (5% *v*/*v*) to all fragments. In all cases, one portion from the same gonad was randomly assigned to each experimental condition. After a 4-hour incubation period under gentle agitation in shaking incubator at 28 °C, media were collected and stored at 4 °C until hormone measurement. Five males and five females were used for each experiment.

2.3.1. In vitro basal steroid release in response to ES

To assess the effect of ES on basal steroid release, gonad explants were incubated with medium supplemented with dimethylsulfoxide

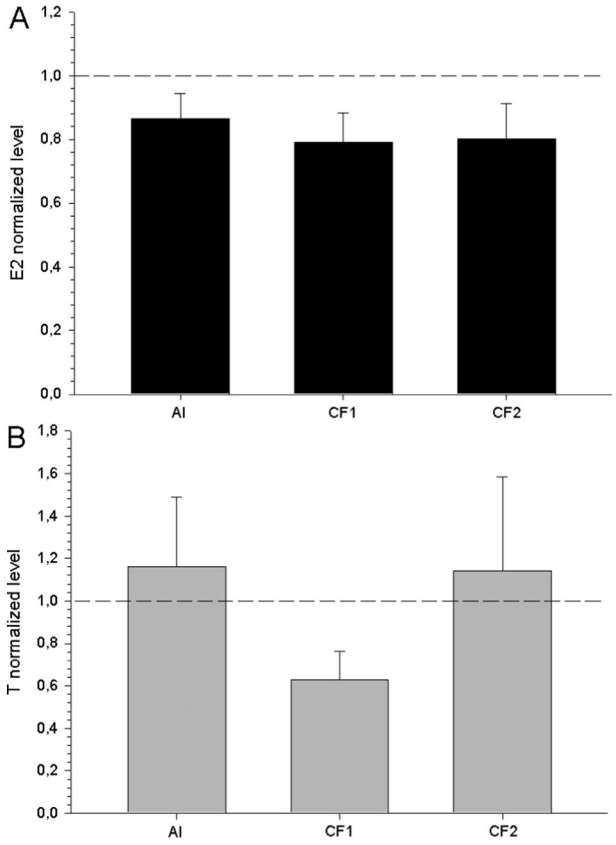

Fig. 1. Basal levels of (A) estradiol (E_2) and (B) testosterone (T) released to the incubation media from *C. dimerus* ovary and testis explants, respectively, following incubation with ES. Data are normalized to the corresponding DMSO control, indicated by the horizontal dashed line, and expressed as mean ± SE ($n = 5$). AI, active ingredient; CF, commercial formulation; DMSO, dimethylsulfoxide.

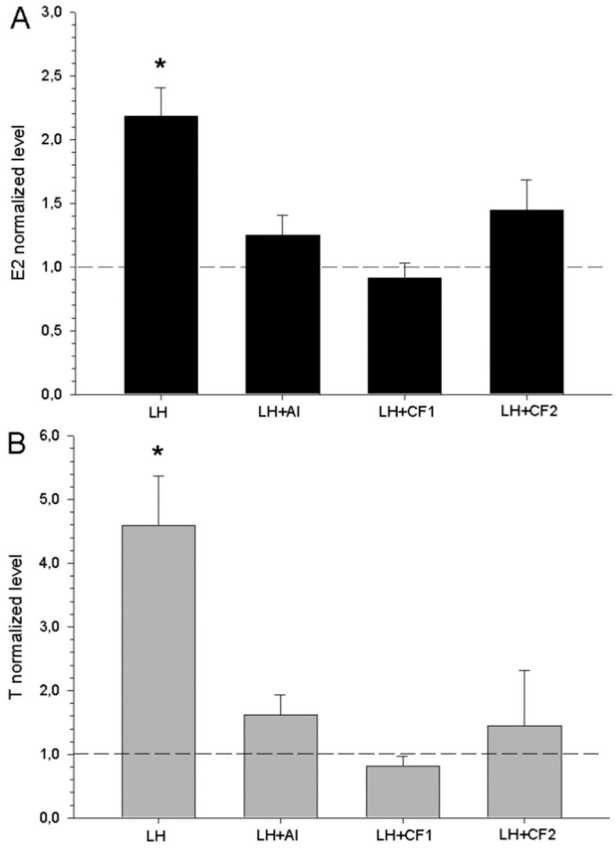

Fig. 2. Endosulfan inhibits LH-stimulated levels of (A) estradiol (E_2) and (B) testosterone (T) released to the incubation media from *C. dimerus* ovary and testis explants, respectively. Data are normalized to the corresponding DMSO control, indicated by the horizontal dashed line, and expressed as mean ± SE ($n = 5$). Asterisk denotes significant differences ($p < 0.05$) with respect to the control using a randomized-block design ANOVA followed by Tukey's multiple comparisons. AI, active ingredient; CF, commercial formulation; DMSO, dimethylsulfoxide; LH, luteinizing hormone.

(DMSO, solvent control), ES-AI (100 μM), ES-CF1 (equivalent to 100 μM of ES-AI) or ES-CF2 (equivalent to 100 μM of ES-AI). The concentration of ES used was chosen to study the mechanism of action of the pesticide based on previous studies *in vitro* as lower doses failed to cause effects and higher doses proved cytotoxic (Bisson and Hontela, 2002).

2.3.2. Effect of ES on LH-stimulated steroid release

The role of ES on steroid release upon stimulation with the gonadotropin LH was evaluated by co-incubating the gonad explants with 100 μM ES (AI, CF1 or CF2) and 0.05 mg/L fish LH. A solvent control (DMSO) and a positive control with LH and DMSO alone were also assayed.

2.3.3. Involvement of the cAMP pathway on ES action

Gonad explants were incubated with DMSO (solvent control) or 1 μM of the adenylate cyclase (AC) inductor forskolin (FK), with or without the addition of 100 μM ES (AI, CF1 or CF2) to elucidate whether ES action occurred up or downstream of AC activation.

2.3.4. Role of steroidogenic enzymes on ES effect

The effect of ES on steroidogenic enzyme activity was evaluated by adding 1 μM of substrates for two key enzymes in the biosynthetic pathway, DHEA (substrate for the enzyme 3-β-hydroxysteroid dehydrogenase) or 17αOHP$_4$ (cytochrome P450 17 *A*1 substrate), alone or in combination with 100 μM ES (AI, CF1 or CF2).

2.4. Steroid levels

Levels of testosterone (T) released from testes and estradiol (E_2) released from ovaries were measured as endpoints in all *in vitro* assays. Testosterone levels were measured by radioimmunoassay (RIA) using a commercial kit (Active® Testosterone RIA DSL44000, Diagnostic Systems Laboratories, Inc. USA); E_2 levels were measured by ELISA (IBL International, Hamburg, Germany). The lower limits of detection for T and E2 were 0.1 ng/mL and 10.6 pg/mL, respectively. Cross-reactivity of the T antiserum with other relevant androgens is 5.8% for 5αDihydrotestosterone, 4.2% for 11-Ketotestosterone and 2.3% for Androstenedione; cross-reactivity of the E2 antibodies is 6.86% for Estrone and 2.27% for Estriol.

2.5. Statistical analysis

Absolute hormone levels were analyzed by means of a randomized block design ANOVA, taking experimental condition as the fixed factor and gonads as the random blocking factor, to control for differences in hormone output between the different gonads. These analyses were followed by Tukey's *post hoc* comparisons test. Data were log transformed when assumptions were not met. Differences were considered statistically significant if $p < 0.05$ (Statistica 7.0 software; StatSoft, Inc., 2004).

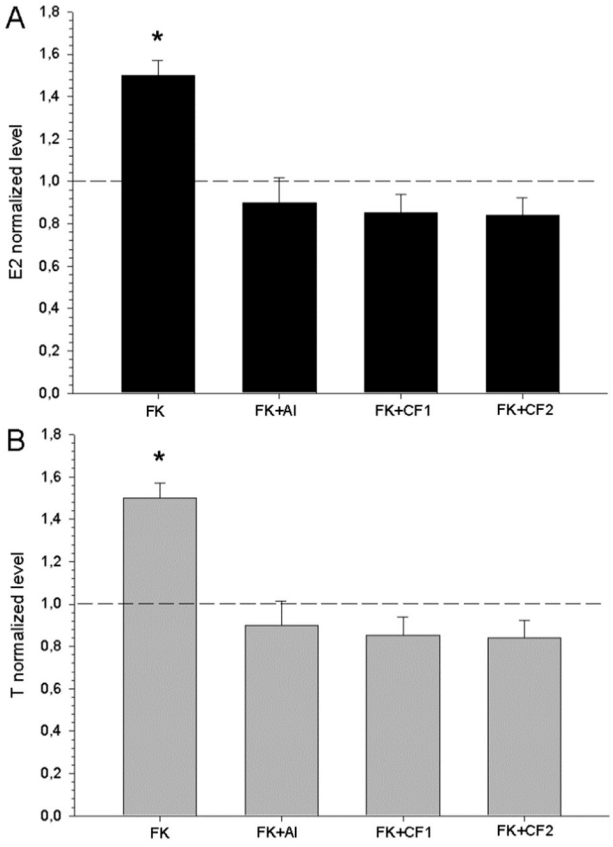

Fig. 3. Stimulation of gonad explants by the AC activator FK. Levels of (A) estradiol (E_2) and (B) testosterone (T) released to the incubation media from *C. dimerus* ovary and testis culture, respectively. Data are normalized to the corresponding DMSO control, indicated by the horizontal dashed line, and expressed as mean ± SE ($n = 5$). Asterisk denotes significant differences ($p < 0.05$) with respect to the control using a randomized-block design ANOVA followed by Tukey's multiple comparisons. AI, active ingredient; CF, commercial formulation; DMSO, dimethylsulfoxide; FK, forskolin.

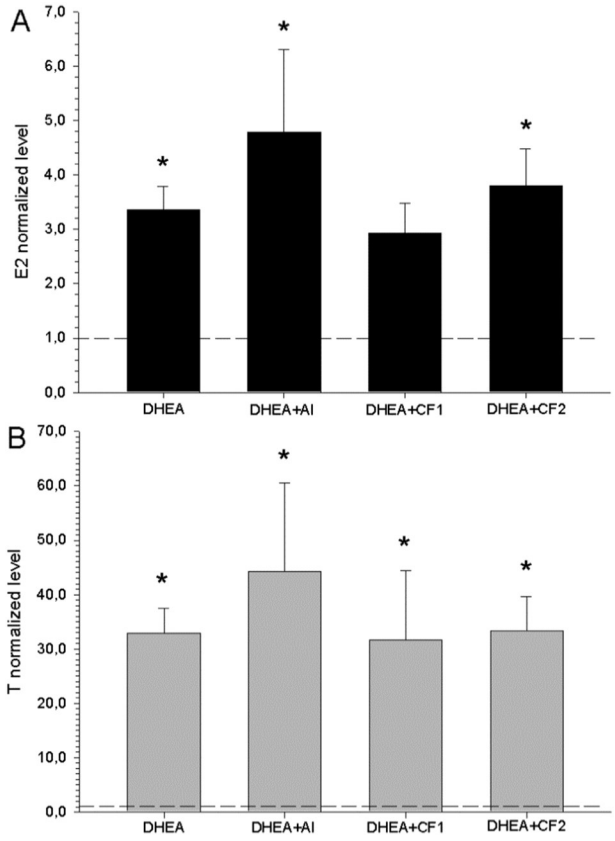

Fig. 4. Stimulation of gonad explants by the 3βHSD substrate DHEA. Levels of (A) estradiol (E_2) and (B) testosterone (T) released to the incubation media from *C. dimerus* ovary and testis culture, respectively. Data are normalized to the corresponding DMSO control, indicated by the horizontal dashed line, and expressed as mean ± SE ($n = 5$). Asterisk denotes significant differences ($p < 0.05$) with respect to the control using a randomized-block design ANOVA followed by Tukey's multiple comparisons. AI, active ingredient; CF, commercial formulation; DHEA, dehydroepiandrosterone; DMSO, dimethylsulfoxide.

3. Results and discussion

Sex steroids are critical for development of the reproductive system and regulation of vertebrate reproduction. In addition to the control of gonad and germ cell development, these hormones are involved in modifying behavior during the reproductive period. Gonadal hormones can exert both stimulatory and inhibitory actions depending on the particular steroid, species, maturation state and target organ (Norris, 2007). Several EDCs are capable of disrupting the gonadal endocrine function by both receptor and non-receptor mediated processes (Villeneuve et al., 2007; Hecker and Giesy, 2008; Yeung et al., 2011).

Endosulfan has been linked to decreased T levels in the broad-snouted caiman (*Caiman latirostris*) exposed *in ovo* (Rey et al., 2009), increased levels of E_2 in waterborne-exposed female catfish (*C. batrachus*; Chakrabarty et al., 2012) and inhibition of ACTH-stimulated cortisol release from rainbow trout adrenocortical cells *in vitro* (*Oncorhynchus mykiss*; Bisson and Hontela, 2002). As it was previously determined that ES was able to disrupt steroidogenesis *in vitro* in gonads of the cichlid fish *C. dimerus* (Da Cuña et al., 2013), in the current study testes and ovaries were cultured with ES in combination with steroidogenic inductors or intermediate precursors in the synthesis of sex steroids in order to elucidate the mechanism of action responsible. Additionally, the effect of the pesticide as a pure active ingredient was compared to that of ES as part of a commercial formulation, in order to examine any possible interaction between ES and other components of the CFs. Formulations have been reported to alter the overall toxicity of AIs. For instance, when comparing the genotoxic effect of pure glyphosate, its surfactant polyethoxylated amine, and their combination in the CF Roundup®, the sum of effects of the components alone was higher than the effect observed with the formulation, indicating an antagonistic effect in the latter (Guilherme et al., 2012). In contrast, the formulation Garlon® showed higher genotoxic potential than the active ingredient triclopyr (Guilherme et al., 2014).

3.1. ES inhibits LH-stimulated steroidogenesis

The ability of the pesticide to provoke changes in basal sex steroids secretion from cultured gonads was studied first. The addition of ES alone, both as AI and CFs, without any steroidogenic stimulus, had no significant effect on basal gonadal steroidogenesis, as measured by sex steroid levels released to the culture media (Fig. 1, $p > 0.05$).

As expected, because LH is one of the tropic hormones (together with FSH) involved in stimulating steroidogenesis *in vivo* (Young et al., 2005), the addition of LH caused a marked increase in both T and E_2 levels detected in the media from cultured testes and ovaries, respectively. Co-incubation of LH and ES (in any of its forms) caused a reduction of sex hormones levels released to the media, when compared to addition of LH alone (Fig. 2). The same effect had been already observed for the AI of ES in our previous study (Da Cuña et al., 2013). Similar results with the AI have been obtained for cortisol secretion stimulated by ACTH in adrenocortical and head kidney cells (Bisson and Hontela, 2002; Hontela et al., 2008). As no differences were

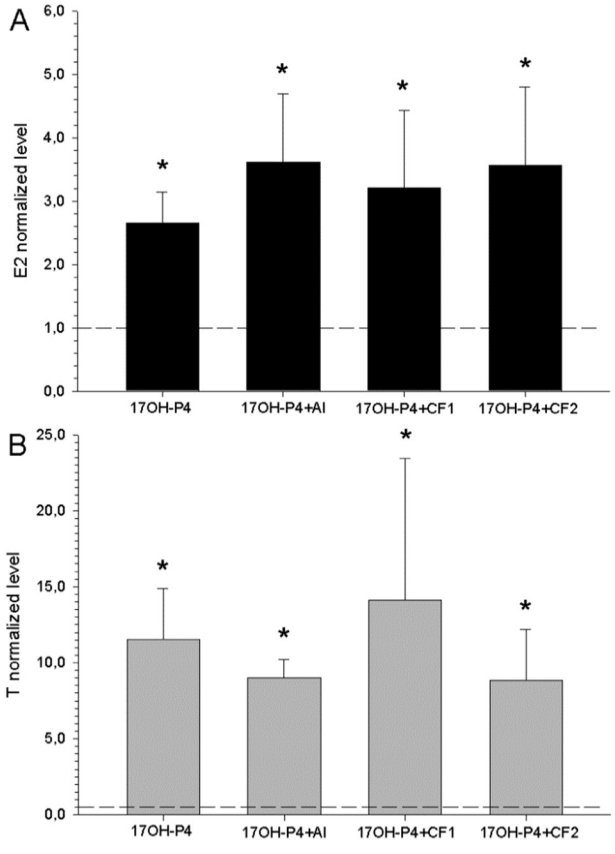

Fig. 5. Stimulation by the P450c17 substrate 17αOHP$_4$. Levels of (A) estradiol (E_2) and (B) testosterone (T) released to the incubation media from *C. dimerus* ovary and testis explants, respectively. Data are normalized to the corresponding DMSO control, indicated by the horizontal dashed line, and expressed as mean ± SE ($n = 5$). Asterisk denotes significant differences ($p < 0.05$) with respect to the control using a randomized-block design ANOVA followed by Tukey's multiple comparisons. 17OH-*P*4, 17αhydroxyprogesterone; AI, active ingredient; CF, commercial formulation; DMSO, dimethylsulfoxide.

observed between the commercial formulations and the active ingredient, the effect can be assumed to be due to ES and not be affected by co-adjuvants.

3.2. Effect of ES on steroidogenesis is not mediated by interference with LH-receptor

In vertebrates, LH has been demonstrated to act through interaction with its cognate LH/choriogonadotropin receptor (LHCGR; Ascoli et al., 2002). In Leydig and follicular cells, LHCGR, member of the superfamily of G protein-coupled receptors, stimulates steroidogenesis mostly via the cAMP/PKA pathway (Chauvigné et al., 2012; Karlsson et al., 2010; Light and Hammes, 2013). EDCs can interfere with hormone receptors acting as antagonists and blocking the action of the endogenous ligand. A variety of pesticides have estrogenic and/or antiandrogenic potential by interacting with the estrogen receptor (ER) or androgen receptor (AR), respectively (Kojima et al., 2004). Affinity of toxicants for non-steroid hormone receptors is less commonly reported, however several phthalates and herbicides have been shown to disrupt thyroid hormone activity (Shi et al., 2012; Sun et al., 2012; Sugiyama et al., 2005).

The AC activator FK was used to test whether ES was acting by antagonizing the effect of LH at the LHCGR level. Both E_2 and T release by cultured gonads was significantly stimulated by FK ($p < 0.05$). As was the case with LH, co-incubation of FK with ES (in any of its forms) resulted in reversion of the effect of the inductor, yielding basal levels of sex steroids in the culture media. Since the effect of ES between AI and CFs was not statistically different ($p > 0.05$), no apparent antagonistic or synergistic effects were observed between ES and co-adjuvants (Fig. 3). Taking into account the results with LH and FK, ES can be assumed to act downstream of LHCGR activation. Similarly, in adrenocortical and head kidney cells of rainbow trout ES AI also acted downstream of ACTH receptor activation in reducing ACTH-induced cortisol secretion, as the same effect was evidenced when incubating with a cAMP analog (Leblond et al., 2001; Bisson and Hontela, 2002).

3.3. ES does not act via inhibition of steroidogenic enzymes 3βHSD and P450c17

Activity and/or expression of steroidogenic enzymes can be modulated by a variety of EDCs: pesticides, herbicides, fungicides, plasticizers and other industrial chemicals (Whitehead and Rice, 2006). Particularly, ES has been shown to decrease mRNA expression of the steroidogenic acute regulatory protein (StAR), responsible for transference of cholesterol from the outer to the inner mitochondrial membrane, and the steroidogenic enzymes 11βHSD2 and 17βHSD12 in male juvenile catfish (*C. batrachus*) following waterborne exposure, even though androgen levels remained unaltered (Rajakumar et al., 2012). Alternatively, in females of the same species, exposure to ES caused increased expression of StAR mRNA and activity of aromatase (CYP19), enzyme in charge of converting androgens to estrogens, leading to an increase in circulating E_2 levels (Chakrabarty et al., 2012). Aromatase activity was likewise stimulated by ES in the human choriocarcinoma cell line JEG-3, without altering CYP19 mRNA expression (Laville et al., 2006).

In the current study, gonads were co-incubated with the pesticide and specific substrates for the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3βHSD) or 17α-hydroxylase/17,20-lyase (CYP17A1 or P450c17), dehydroepiandrosterone (DHEA) and 17αhydroxyprogesterone ($17\alpha OHP_4$), respectively, to study the effect of ES on the activity of key steroidogenic enzymes. Since both steroids act as precursors in the biosynthetic pathway of both T and E_2, levels of these hormones were significantly increased by the addition of the precursors to the culture media ($p < 0.05$; Figs. 4, 5). Addition of ES (in any of its forms) did not significantly alter hormone levels when compared with enzyme substrates alone ($p > 0.05$), indicating that the pesticide is neither interfering with the activity of the studied enzymes, nor with other enzymes downstream the sex steroid synthetic pathway, such as aromatase or 17βHSD. Contrary to the present study, Moses Inbaraj and Haider (1988) found decreased activity of 3βHSD in follicular cells of female spotted snakehead (*Channa punctatus*) upon exposure to waterborne ES. A decreased expression of P450c17 was reported by

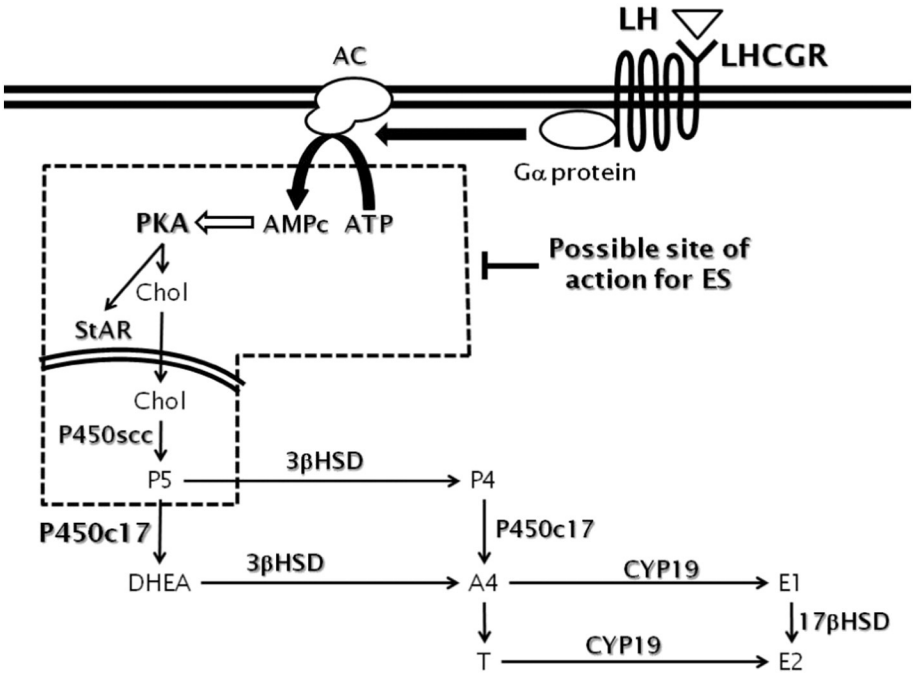

Fig. 6. Schematic diagram of the steroidogenic pathway in gonads of most teleost fish (for review see Schulz et al., 2001). Binding of LH to LHCGR promotes activation of PKA which in turn increases active StAR levels. Cholesterol is transferred to the inner mitochondrial membrane where it is cleaved by P450scc to pregnenolone. Synthesis of T and E2 follows, by effect of the steroidogenic enzymes 3βHSD, P450c17, 17βHSD and/or CYP19. Dotted line encloses the possible sites of action of ES in inhibiting LH-stimulated steroid release, based on the current results in *C. dimerus*.

Rajakumar et al. (2012) when male juvenile catfish (*C. batrachus*) were exposed to ES, though this was not enough to alter plasma T levels, suggesting that altered enzyme levels do not necessarily result in altered product hormone levels.

4. Conclusion

Based on the results of the current study, the steps of the endocrine regulation of steroidogenesis affected by ES in both testes and ovaries appear to be located downstream of adenylate cyclase activation and upstream of pregnenolone conversion to progesterone and/or dehydroepiandrostenedione (Fig. 6). Because no differences were found between the active ingredient alone or in combination with excipients in either commercial formulation, the observed effect on steroidogenesis is caused by ES itself, and not by any of the remaining components of the commercial mixtures. The concentration of ES responsible for the effects observed is above those found in fish tissues (high end of 1 μM), however since sex steroids play a crucial role in reproduction and therefore small changes in their levels could lead to reproductive failure, the obtained results provide a further approach for understanding the impact of an ecologically relevant endocrine disruptor on fish reproductive function.

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