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REVIEWS

Effects of endocrine disruptors on reproduction in viviparous teleosts with intraluminal gestation

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Abstract Many water systems worldwide are affected by pollutants, including potential endocrine disruptors (EDs). Most studies on the effects of EDs on fish reproduction have focused on oviparous species. However, some important groups of fishes are not oviparous and there is scarce information about how EDs affect species with alternative reproductive modes. Goodeinae is a viviparous matrotrophic subfamily with intraluminal gestation (IG), where transfer of nutrients occurs and embryos develop inside the ovarian cavity. Goodeinae is endemic to the Mexican Central Plateau, an area affected by potential EDs, including 2,4-dichlorophenoxyacetic acid (2,4-D). This review synthesizes the available information about EDs in viviparous teleosts with IG and performs a case study on the effects of 2,4-D on gonadal structure of two Goodeinae species. We hypothesized that individuals exposed to 2,4-D might show altered gonadal structure. The available information included effects on gene expression, sexual differentiation, gametogenesis, secondary sexual characteristics, and sexual behavior. Knowledge gaps persisted on the effects of EDs on viviparous teleosts with IG. Holistic approaches are needed to understand the mechanisms underlying endocrine disruption effects. Field studies are needed to evaluate the consequences of EDs on wild populations. The case study revealed histological alterations in oocytes, spermatogonia, and sperm cysts in fishes exposed to 2,4-D. Ultrastructurally, gonads exhibited alterations in oocyte mitochondrial and nuclear membranes, and in spermatid mitochondria. The observed changes could be related to 2,4-D exposure, which may affect species reproduction in their natural environment. Matrotrophic viviparous teleosts with IG may serve as models to explore endocrine disruption.

Keywords Endocrine disruptors · Viviparity · Goodeidae · Reproduction · Gonadal histological alterations · Gonadal ultrastructural damage

Introduction

Today, many water systems around the world are affected by contaminants from human activities, mainly agricultural practices in which pesticides are used indiscriminately. These pollutants are released into the atmosphere and finally reach water bodies,

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where they may exert adverse effects on aquatic organisms, including various fish species (Favari et al. 2002). Many of these pesticides have been listed as potential endocrine disruptors (EDs), a wide range of chemicals that affect the endocrine system, including the hypothalamic–pituitary–gonadal axis, with subsequent adverse effects on reproduction (Mills and Chichester 2005; Vandenberg et al. 2012). Because of these effects on fish, several teleost fish species have been used as indicators of water quality (Schiedek et al. 2006; Hedman et al. 2011). Nevertheless, most studies of the effects of pesticides on fish reproduction have focused on oviparous species (Hutchinson et al. 2000; Ruggeri et al. 2008; Genovese et al. 2014). Whilst most fish species are oviparous, many fish that are ecologically or economically important exhibit alternative modes of reproduction. Some fish such as the Western mosquitofish *Gambusia affinis* and the guppy *Poecilia reticulata* are viviparous bearing live young. Viviparous teleosts could be informative models on the effect of EDs on reproduction because they possess some reproductive characteristics that may function as endpoints of the effects of EDs. These characteristics include ovaries that serve as the site of oocyte production and gestation, internal fertilization, sexual dimorphism, and placental analogues that enable metabolic exchange between mother and embryo (Wourms 1981; Wourms et al. 1988; Uribe et al. 2005; Blackburn 2015; Schindler 2015). There is some evidence that EDs affect reproductive aspects like gametogenesis, sexual differentiation, sex ratio, development of secondary sexual characteristics and the reproductive behavior of viviparous fish species (Larsson et al. 2000; Dreze et al. 2000; Rasmussen and Korsgaard 2004). These effects have been observed at various levels of biological organization: molecular, cellular, tissues, individuals, and populations (Rotchel and Ostrander 2003; Edwards et al. 2010; Bergek et al. 2012; Dang 2014).

In viviparous teleosts, gestation can take place inside the ovarian cavity (intraluminal gestation, IG), and has been reported in most families with live-bearing species, including Zoarcidae (the eelpout *Zoarces viviparus*) and Goodeidae (the blackfin goodeid *Girardinichthys viviparus* and the yellowfin fish *Girardinichthys multiradiatus*). Alternatively, gestation can occur inside the ovarian follicle (intrafollicular gestation), as reported in species of Poeciliidae (*P. reticulata* and the swordtail *Xiphophorus helleri*). Additionally, viviparous fish exhibit two types of nutritional patterns: lecitotrophy and matrotrophy. In the former, embryos take the nutrients exclusively from the yolk, like in most species of Poeciliidae (Wourms et al. 1988); in the latter, the mother supplies nutrients from various sources, different from the yolk reserve, as in species of Zoarcidae and Goodeinae (Wourms et al. 1988). Among viviparous teleosts, those that exhibit IG are particularly interesting for studying the effects of EDs. In addition, due to their matrotrophic nature, these teleosts display placental analogues, and all embryos are immersed in the ovarian fluid (Schindler 2015). The ovarian fluid contains substances secreted by the ovarian internal epithelium, and may contain substances from the environment. For example, EDs can cross the ovarian internal epithelium, and they can be taken up by the embryos (Wourms et al. 1988; Schindler 2015).

The Goodeinae (a viviparous matrotrophic subfamily of Goodeidae), which are endemic to Mexico, are highly important and interesting, from the perspectives of ecology, evolution, biogeography, and conservation (Domínguez-Domínguez et al. 2005). These species are constantly threatened by exposure to water contaminants. Their main area of distribution, the Mexican Central Plateau, is highly populated, and it supports intensive agricultural activities and pesticide use (Favari et al. 2002; De la Vega-Salazar and Macías-García 2005). Constant exposure to aquatic pesticides may compromise the reproductive capacity of these species and their stability in the wild (Arellano-Aguilar and Macías-García 2008). However, no baseline toxicity data are available for the Goodeinae, regarding potential EDs and their effect on reproduction.

The objective of this review was to analyze and synthesize the available information about the potential effects of EDs on the reproductive features of viviparous teleosts with IG. We included information collected at different levels of biological organization (e.g., molecular, cellular, tissue, individual, and population). We focused on studies conducted on *Z. viviparus*, of the Zoarcidae family, and studies conducted on *Girardinichthys viviparus* and *G. multiradiatus*, of the Goodeinae subfamily (Rasmussen et al. 2002; Vega-López et al. 2006; Arellano-Aguilar and Macías-García 2008). Information on the effects of EDs on the reproduction of these species was available

from laboratory studies and from field studies conducted in polluted areas. We identified the gaps in that research, and we proposed a research direction for this topic. We also conducted a case study to analyze two Goodeinae species, *Goodea atripinnis* (Blackfin Goodea) and *Chapalichthys encaustus* (Barred splitfin), to assess the acute toxicity (LC_{50}) of a potential ED, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). We analyzed the resulting histological and ultrastructural changes in the gonads of female and male adults after 2,4-D exposures. Due to the continuous and increasing pollution of water bodies with EDs, there is an urgent need to address the knowledge gaps in this topic.

Endocrine disruptors during pregnancy in viviparous fish with intraluminal gestation

Exposure of gestating females to EDs may have effects on the progeny at different levels. Considering the characteristics of viviparous teleosts with IG, substances may be transferred from the mother to the embryos (matrotrophy). The inner ovarian epithelium functions as the maternal component of the placental association. It secretes embryotrophic liquid into the ovarian cavity, which can be absorbed by the embryo through different organs that provide surfaces for nutrient absorption (Schindler 2015). EDs can cross the inner ovarian epithelium and can be absorbed by the embryos; thus, they might affect the maternal-fetal relationship, embryonic development, and the expression of genes (e.g., the vitellogenin gene; Korsgaard et al. 2002; Rasmussen et al. 2002; Brande-Lavridsen et al. 2013).

Placentotrophy in viviparous teleost fish

Viviparous teleosts undergo placentotrophy, the intimate apposition or merger of fetal organs with maternal (or paternal) tissues for physiological exchange (Mossman 1937; Blackburn 2015). In viviparous fish, the term *placental analogue* refers to the interaction between maternal and embryonic tissues for metabolic exchange. Substances are transferred through the placental analogue from the mother to the embryo, similar to mammals (Wourms et al. 1988; Blackburn 2015; Schindler 2015). In viviparous teleosts, gestation occurs in the ovary (intraovarian gestation) due to the absence of oviducts (Wourms 1981). The ovarian tissue in matrotrophic species is specialized for the provision of nutrients. In species that undergo IG, including members of the Goodeidae and Zoarcidae families, the epithelium lining the ovarian lumen secretes embryotrophe, a nutritive fluid within which embryos develop. The embryo ingests these nutrients or absorbs them through permeable structures such as the yolk sac, the pericardial sac, the gill epithelium, or the trophotaeniae (embryonic hindgut extensions projecting outward from the perianal region that are specialized for nutrient absorption (Wourms et al. 1988; Schindler 2015). The apposition of these structures with the inner ovarian epithelium forms a trophotaenial placenta, which serves as the major passage for nutrients in Goodeids (Wourms et al. 1988; Wourms 2005; Schindler 2015). In *Xenotoca eiseni* (Goodeidae), maternal serum proteins are transferred into the embryotrophe to nourish the embryo (Schindler and Hamlett 1993; Schindler 2015). Given these characteristics, viviparous teleost with IG can serve as models of the effects of exposure to EDs on maternal-fetal relationships, embryonic development and fetal health in viviparous vertebrates. According to Edwards et al. (2010), viviparous fish may constitute a complementary system to mammals to assess the effects of contaminants on postnatal health.

Transfer of pollutants from mother to embryo

The evidence that substances are transferred from mother to embryo in matrotrophic species implies that the mother can also transfer other substances present in her environment that can be absorbed by various embryonic structures. The absorption of potentially EDs may affect embryonic development in general (growth, development of the skeletal system) and may particularly affect sexual determination, sexual differentiation, gonadal development, the sex ratio of offspring, and the overall reproductive capacity of individuals when they reach sexual maturity.

Information on the effects of xenobiotics with the potential for endocrine disruption on the maternal-embryonic relationship in viviparous teleosts with IG is limited. The species most studied in this respect is *Z. viviparus*. In this species, some chemical pollutants (octylphenol, tributyltin, and nonylphenol)

were transferred from mother to embryo when they were injected intraperitoneally into pregnant females (Rasmussen et al. 2002; Brande-Lavridsen et al. 2013). In the same species, 4-tert-octylphenol (4-tOP) was detected in ovarian fluid, which was absorbed by embryos. Contaminants have been detected in developing embryos and can be bioaccumulated at high concentrations because early-stage embryos are less able to metabolize and excrete compounds (Sharpe et al. 2010; Brande-Lavridsen et al. 2013).

In species of Goodeinae there is a gap in the information on the effect of pollutants during pregnancy. All species of this subfamily are viviparous matrotrophic (Schindler and Hamlett 1993; Schindler 2015). Hence, in the case of pregnant females exposed constantly to aquatic pesticides, the transfer of substances from mother to embryo may include the transfer of pollutants into the developing offspring, as is reported in *Z. viviparus* (Brande-Lavridsen et al. 2013).

Effects of endocrine disruptors on embryonic development

Exposure to EDs may affect maternal-fetal trophic relationships in viviparous teleost fish. In *Z. viviparus*, exposure to the EDs ethinyl 17α-estradiol, 17β-estradiol, and 4-tOP depleted calcium from the ovarian fluid and restricted amino-acid availability in the maternal plasma (Korsgaard et al. 2002; Rasmussen et al. 2002). Thus, prolonged exposure to these substances can affect the growth of developing embryos by altering the distribution of calcium between plasma and ovarian fluid (Rasmussen et al. 2002). In laboratory studies, exposure to 17β-estradiol at dosages similar to 17β-estradiol concentrations detected in the environment (5.7–133 μg/L), caused embryonic malformation, and the reduction of ovarian fluid. According to those authors, the observed malformations may be due to reduced calcium availability and reduction of the amount of ovarian fluid (Morthorst et al. 2014). In mammals, the absence of amniotic fluid is known to cause defects in embryos (Kaufman and Chang 2000). Fluid depletion likely results from effects on water-transport proteins such as the aquaporins, occludins, and claudins, which are under the control of steroid hormones (Nicholson et al. 2010).

Vitellogenin, a molecular marker of endocrine disruption in embryos

In embryos, vitellogenin is used as a marker of endocrine disruption at the molecular level because estrogen influences vitellogenin expression (Sumpter and Jobling 1995). Vitellogenin mRNA was present in *Z. viviparus* embryos from females exposed to 4-tOP (100 μg/L), which displays estrogenic activity, demonstrating that a concentration of 100 μg/L of 4-tOP was sufficient to prompt an estrogenic response (Rasmussen et al. 2002). However, at concentrations similar to those found in the environment (14 μg/L), no vitellogenin mRNA expression was detected (Rasmussen et al. 2002). According to those authors, vitellogenin is not required during the embryonic stage of the fish's life cycle, and thus its expression involves an energy expenditure that may negatively affect embryonic growth. In nature, such effects may have consequences at the population level because exposed offspring are smaller and suffer decreased performance (Rasmussen et al. 2002).

Effects of endocrine disruptors in sexual determination, sexual differentiation and sex ratio

Steroid hormones play an important role in sexual determination and differentiation in teleosts fish (Devlin and Nagahama 2002; Guerrero-Estévez and Moreno-Mendoza 2010); hence, it is very likely that chemical contaminants with steroid-like activity may alter these processes. The steroid system used by fish is complex and differs in some aspects from other vertebrates; besides testosterone, 11-ketotestosterone is also produced by fish and is usually more effective in stimulating the development of secondary sexual characteristics, reproductive behavior and spermatogenesis (Borg 1994). Laboratory studies have shown that embryos from *Z. viviparus* females exposed to 4-tOP (65–100 μg/L) exhibited abnormal testicular development in which a cavity is formed, similar to that observed normally in female gonads. However, concentrations of 4-tOP similar to those reported in the environment (14 μg/L) caused no obvious abnormalities in gonadal structure (Rasmussen et al. 2002). In situ hybridization revealed the presence of the estrogen receptor in embryonic gonads of this species, suggesting that the observed effects may be mediated

by direct interaction between xenoestrogens and gonadal estrogen receptor (Rasmussen et al. 2002).

On the other hand, sex-ratio deviation reflects the effect of EDs at the population level (Edwards et al. 2010). In *Z. viviparus,* the sex ratio of offspring tended toward a greater number of males in populations from contaminated sites with EDs compared with populations in reference sites, where the sex ratio is nearly 1 male: 1 female (Larsson et al. 2000; Larsson and Förlin 2002). Further, simulations of the extinction probability of *Z. viviparus* populations revealed that populations consisting of 38.7 % females may decrease to 5 % of their original size over 100 years (Hanson et al. 2005; Edwards et al. 2010). In species of Goodeinae there is a lack of information on this topic.

Effects of endocrine disruptors on gene expression, gametogenesis and secondary sexual characteristics

In adult fish, endocrine disruptions have effects on reproduction that range from gene expression to sexual behavior. In this regard, vitellogenin is the most studied gene; indeed, vitellogenin is considered a molecular marker of endocrine disruption (Rasmussen et al. 2002; Vega-López et al. 2006). Gametogenesis and gonadal structure are endpoints of endocrine disruption effects at the cellular and organ levels. Thus, EDs affect the health of individuals (Velasco-Santamaría et al. 2010; Brande-Lavridsen et al. 2013). On the other hand, impairments in secondary sexual characteristics and sexual behavior are endpoints at the individual level, which may affect the reproduction of populations (Arellano-Aguilar and Macías-García 2008).

Effects on gene expression

Few studies have focused on changes in gene expression in viviparous fish, which may provide insight into the genetic mechanisms of the effects of contaminant exposure. Such research would be of great importance for evaluating genomic responses to contaminant exposure. These investigations require the characterization of genes that can be targeted by EDs. In the National Center for Biotechnology Information (NCBI) database, sequences of genes related to reproductive function that can potentially be targeted by EDs in viviparous fishes with IG, are available for the genes encoding vitellogenin (*G. viviparus* and *Z. viviparus*) and estrogen receptor alpha (*Z. viviparus*).

One of the most studied genes in endocrine disruption responses at the molecular level is the vitellogenin gene. The vitellogenin is a natural component of the yolk found normally in mature females (Selman and Wallace 1983). The presence of vitellogenin in males is used as a biomarker of estrogen exposure (Sumpter and Jobling 1995).

Exposure of *Z. viviparus* females to 4-tOP increases the mRNA expression of vitellogenin, which indicates that this compound exerts an estrogenic activity (Rasmussen et al. 2002). Similarly, exposure to EE2 causes an increase in vitellogenin concentration in the plasma and up-regulates vitellogenin and estrogen receptor mRNA expression in this species (Velasco-Santamaría et al. 2013). Vitellogenin was detected in the plasma of *G. viviparus* males that were exposed to polychlorinated biphenyls (Vega-López et al. 2006) and in the plasma of *Z. viviparus* males that were exposed to 4-tOP (Rasmussen et al. 2005). In *Z. viviparus*, exposure to 17β-estradiol at dosages similar to concentrations of this hormone detected in the environment (5.7–133 μg/L) caused elevated concentrations of plasma vitellogenin in adult females (Morthorst et al. 2014). On the other hand, 4-tOP exposure induced expression of the hepatic estrogen receptor mRNA in *Z. viviparus* (Andreassen et al. 2005). Field studies of *G. viviparus* populations from polluted lakes that contained xenoestrogens revealed vitellogenin in the plasma of males (Olivares-Rubio et al. 2015). Those authors found that males of *G. viviparus* were particularly sensitive to EDs, which could be related to the population decline.

Effects on gametogenesis and gonadal structure

The EDs act by disrupting the hypothalamic-pituitary–gonadal axis, with consequences for both male and female gametogenesis (Scholz and Klüver 2009). Sperm count is a readout for spermatogenesis; particularly, Goodeids are good models for assessing and quantifying spermatogenic capacity because they release sperm into spherical structures called spermatozeugmata (Grier et al. 1978; Edwards et al. 2010). Exposure to the estrogenic compounds, 4-tOP and 17β-estradiol, led to degeneration of the lobular structure of testes and fibrosis in the interstitial tissue of male *Z. viviparus* (Rasmussen et al. 2005). Similarly, EE2 dramatically disrupted spermatogenesis in

Z. viviparus; exposure to this ED caused marked interstitial fibrosis, germinal cell necrosis, and reductions in the numbers of spermatocytes and spermatogonia in the cyst (Velasco-Santamaría et al. 2010). Several natural populations of *Z. viviparus* have displayed changes in reproductive success. Laboratory tests, where gestating females were exposed to the EDs present in their environments, suggested that some of these compounds affected the reproductive capacity of the offspring by inducing the development of ovotestes in males (Gercken and Sordyl 2002; Brande-Lavridsen et al. 2013). In this species, 4-tOP exposure led to alterations in the structure of Sertoli cells (Rasmussen and Korsgaard 2004). One of the most damaging effects of EDs was their capacity to reduce reproduction by compromising the production of gametes (Arellano-Aguilar and Macías-García 2008).

On the other hand, changes in the gonadosomatic index reflected responses to EDs at the level of individuals. For example, in *Z. viviparus*, decreases in the male gonadosomatic index have been reported after exposure to octylphenol (Rasmussen and Korsgaard 2004) or EE2, either alone or in combination with 17β-trenbolone (Velasco-Santamaría et al. 2010).

Effects on secondary sexual characteristics and sexual behavior

Viviparous teleost species are sexually dimorphic, making them good models for studying the effect of EDs on secondary sexual characteristics. One of these sexually dimorphic characters is the gonopodium, a modification of the male anal fin that participates in sperm transfer (Downing-Meisner 2005). Similarly, courtship behavior and the development of ornaments are particularly informative when evaluating exposure to EDs. These external markers enable the use of noninvasive methods to assess sex ratio, time to puberty, female masculinization, and male feminization. Masculinization of the anal fin in females and development of the gonopodium are organ-level responses that can be evaluated to assess environmental exposure to contaminants (Edwards et al. 2010).

Exposure of *G. multiradiatus* to sublethal doses of methyl parathion during embryonic development led to alterations in the development of ornaments in dorsal and anal fins when these individuals reach sexual maturity; therefore, the availability of attractive males within populations was reduced (Arellano-Aguilar and Macías-García 2008). Similarly, prenatal exposure to this pollutant caused differences in the sexual behavior of males, with exposed males displaying a more parsimonious style of courtship (Arellano-Aguilar and Macías-García 2008). According to those authors, these differences in behavior may be due to an overall decrease in metabolism or to permanent damage to the central nervous system. Those authors also state that exposed males were less attractive to females, significantly decreasing the chance of mating and thus reducing the overall fitness of the exposed population. Methyl parathion is found in the natural habitat of this species; therefore, the survival of exposed species may be threatened due to its direct link to sexual selection, which is intense in this species (Macías-Garcia et al. 1998).

Case study of two Goodeid species: Effects of 2,4-D on structure and ultrastructure of the gonad of *Goodea atripinnis* and *Chapalichthys encaustus*

Most of the aquatic systems of the Mexican Central Plateau, the main range of the viviparous Goodeids, are affected by the presence of pesticides. One of these waterbodies is Lake Chapala, where organochlorine compounds such as 2,4-D herbicide are widely used and has been reported in the lake (Arévalo-Hernández et al. 2011). In Lake Chapala several viviparous Goodeid species are distributed, among them are *G. atripinnis* and *C. encaustus* (Domínguez-Domínguez et al. 2005). The herbicide 2,4-D has been reported as a potential endocrine disruptor (Mnif et al. 2011). In rainbow trout, 2,4-D shows estrogenic activity, increasing levels of plasma vitellogenin (Xie et al. 2005). In *Xenopus* sp. 2,4-D inhibits oocyte maturation (Stebbins-Boaz et al. 2004; LaChapelle et al. 2007). The aim of this case study was to determine the LC_{50} of 2,4-D for *G. atripinnis* and *C. encaustus* and evaluate the possible sublethal effects of this herbicide in the structure and ultrastructure of the gonad of these two endemic species of Mexico, which have different distribution and tolerance to environmental conditions.

Materials and methods

Test organisms

Individuals of *G. atripinnis* and *C. encaustus* were maintained and bred in aquaria, under standard

conditions, temperature: 25 ± 1 °C, and photoperiod 12 h light: 12 h dark, and were fed with commercial flakes (Wardley, The Hartz Mountain Corporation). Temperature, hardness, pH and dissolved oxygen were recorded periodically. The fish were maintained for 7 days in 40 L aquaria with tap water under standard conditions (previously mentioned) for acclimatization before the beginning of the toxicity tests (APHA et al. 2005). The parameters were adjusted to pH at 7.5 and hardness at 40 mg/L of $CaCO_3$. The oxygen saturation levels were maintained by minimal and constant aeration. Individuals of experimental and control batches were anesthetized with a solution of sodium bicarbonate ($NaHCO_3$) (2 %) and immediately sacrificed by decapitation. The procedures were performed in accordance with the Mexican Official Guidelines NOM-062-ZOO-1999 (SAGARPA 2001).

Determination of LC_{50}

To determine the LC_{50} static tests were performed for 96 h with juveniles of *G. atripinnis* and *C. encaustus* separately. A total of five groups, each with four fish, were exposed to different concentrations of the commercial formulation of the herbicide 2,4-D, Hierbamina (dichlorophenoxyacetic acid amine salt, Syngenta). Since there were not available data on LC_{50} for Goodeidae species, a preliminary range-finding test was conducted to find the range of concentrations. Different nominal concentrations (5) were tested from 200 to 240 mg/L (200, 210, 220, 230 and 240 mg/L) for *G atripinnis* and from 100 to 180 mg/L (100, 120, 140, 160 and 180 mg/L) for *C. encaustus*. Mortality was recorded. A fish was considered dead when it did not respond after gentle prodding with a glass rod; dead fish were removed from the tank immediately. Fish behavior was observed during the exposure. All bioassays included a control group and were performed in triplicates. The control medium was tap water. The LC_{50} values and their corresponding confidence limits at 95 % were calculated by the Probit program (XLStat 2014, Addinsoft). Subsequently, acute assays (96 h) were performed. Two batches each of four individuals were exposed to concentrations of 143.35 mg/L (1/1.45th of LC_{50} value) and 230.96 mg/L (1/0.9th of LC_{50} value) for *G. atripinnis*, and 94.06 mg/L (1/1.45th of LC_{50} value) and 133.72 mg/L (1/1.02th of LC_{50} value) for *C. encaustus*. A third group for each species was used as control (no herbicide) and all assays were performed in triplicates.

Determination of 2,4-D during testing

High-performance liquid chromatography (HPLC) (Agilent technologies, model 1260 Infinity) was used for determinations of the presence and concentration of 2,4-D in the aquarium water for toxicity tests. The determination of 2,4-D was by HPLC with a modification of the method employed by Behbahani et al. (2014). We employed a commercial formulation for the toxicity assay, Hierbamina, which is highly water soluble. We conducted a standardization with the 2,4-D standard reagent (Fluka, Sigma-Aldrich) in order to calculate the actual concentration of 2,4-D present in the toxicity tests. The mobile phase was formed by a 60 % of phase A (water: acetic acid 1 %) and 40 % of phase B (Acetonitrile: acetic acid 1 %). We employed a Zorbax SBC18 column (5 µm of pore diameter and 150 × 46 mm length). The flow rate was 1 mL/min. The limit of detection of the method was 1 mg/L. The detection of 2,4-D was performed at the wavelength of 283 nm. All reagents were HPLC grade. In order to calculate the percentage of 2,4-D available at the end of acute tests, water samples from exposure aquaria were taken at the beginning (0 h) and at the end (96 h) of the essays at five different concentrations (100, 120, 200, 210 and 220 mg/L). The samples were taken in duplicates.

Histological and ultrastructural analysis

Gonads of individuals from experimental and control batches were fixed with 2.5 % glutaraldehyde in posphate buffered saline solution (PBS, 0.05 M) for two hours, washed with PBS, post-fixed with 1 % Osmium tetroxide (OsO_4), dehydrated in gradual alcohol series and embedded in resin. Semithin sections of 2 µm were obtained and stained with Toluidine blue and analyzed by light microscopy (modified from Guerrero-Estévez and Moreno-Mendoza 2012). Thereafter, thin sections were obtained and were contrasted with uranyl acetate and lead citrate (Guerrero-Estévez and Moreno-Mendoza 2012), and analyzed by transmission electron microscope (TEM, JEOL). Images of histology and ultrastructure of the gonads to evaluate the possible alterations after exposure to 2,4-D were obtained.

Histological images were obtained in a light microscope Karl Zeiss. We followed the Guidance document on the diagnosis of endocrine-related histopathology in fish gonads (Braunbeck et al. 2010) for the description of histological changes. We took into account the diagnostic criteria.

Statistical analysis

In the histological analysis, we recorded the frequency of changes in ovaries and testes observed in exposed and control fish, for each studied species. The severity of 2,4-D-induced changes observed in the gonads of fish was semi-quantitatively evaluated with the grading scale proposed by Braunbeck et al. (2010). Severity grading employed the following system: Not remarkable (no findings associated with a particular diagnostic criterion), Grade 1 (minimal change, fewer than 2 occurrences per section), Grade 2 (mild change, 3–5 occurrences per tissue section), Grade 3 (moderate change, 6–8 occurrences per tissue section), and Grade 4 (severe change, more than 9 occurrences per tissue section).

We also calculated a Histopathological Index, based on the formula proposed by Bernet et al. (1999): $\sum = (a \times w)$, where w = importance factor, and a = score value. The atresia index (Iatr) and follicle cell hypertrophy index (Ifh) were calculated for females, and the plasma alteration index (Ipa) and structural alteration index (Ist) were calculated for males. Those authors classified the alterations into three increasing importance factors (1–3). Taking into account that atresia is an irreversible process, the importance factor was 3. According to those authors, the importance factor for follicle cell hypertrophy, plasma alteration and structural alterations was 1. The score value (a) ranged from 0 to 6 depending on the degree of the alteration. A Kruskal–Wallis test was carried out to detect differences, using a $p \leq 0.5$ level of significance.

Results

LC_{50} of 2,4-D and detection of 2,4-D during toxicity assays

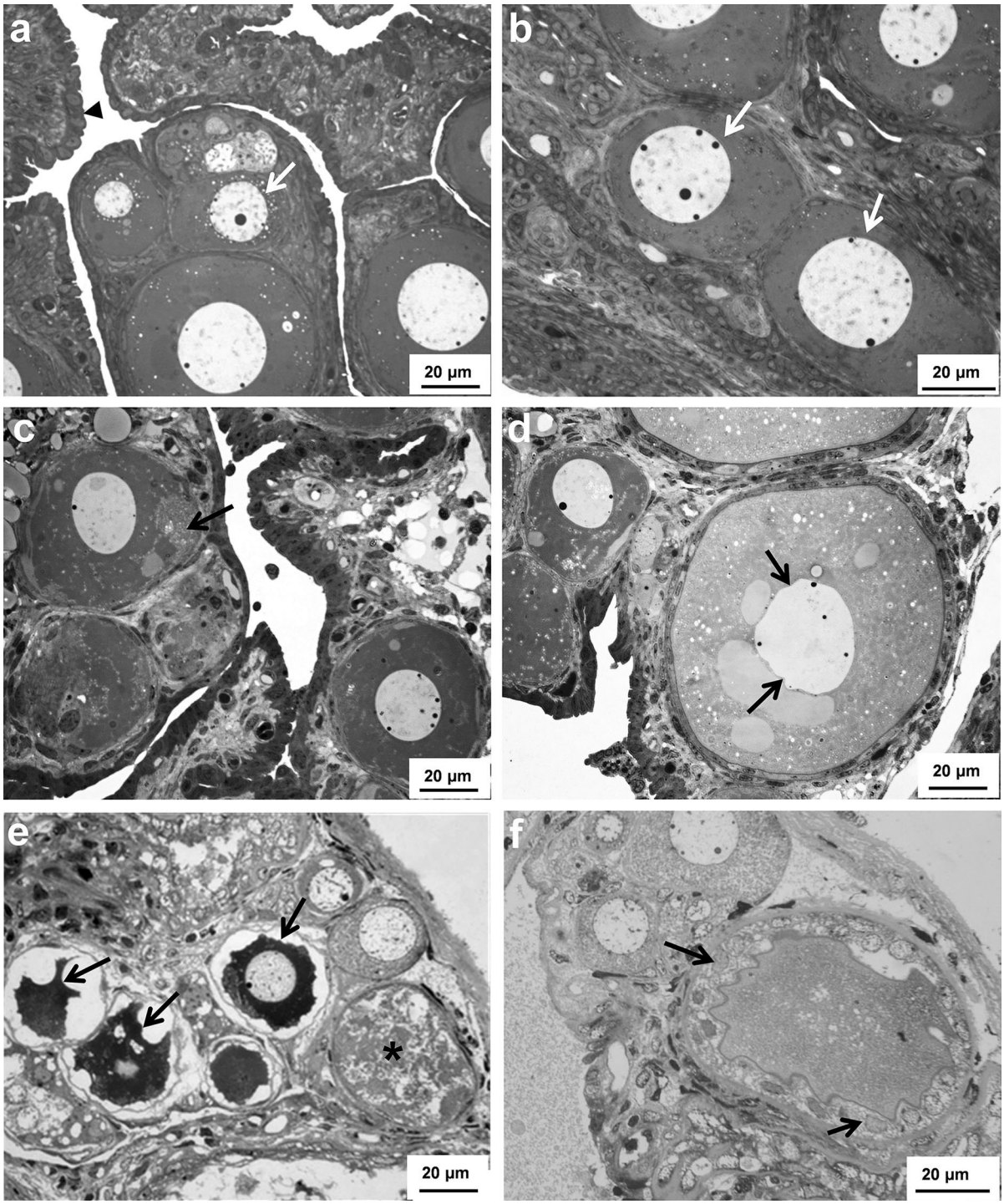
The LC_{50} (96 h) for *G. atripinnis* was 207.87 mg/L (confidence limit: 203.72–211.93) and for *C. encaustus* was 136.40 mg/L (confidence limit: 126.98–146.68) (Online Resource 1). The equation obtained as a result of the standardization of Hierbamina with the standard reagent was $y = 0.9532x$, with $R^2 = 0.998$. We detected the 2,4-D at the end of the acute tests, a 95.83 % of the initial concentration remained on average (Online Resource 2). All the samples showed a similar tendency.

Fig. 1 Light microscopy of ovarian tissue of control and exposed females of *Goodea atripinnis*. **a**, **b** Sections of control ovary. **a** Oocytes at chromatin-nucleolar stage (*arrow*), the internal ovarian epithelium (*arrowhead*) lines the ovarian cavity. **b** Oocytes at perinucleolar stage (*arrows*). High-quality ooplasm and nuclei are observed. **c**, **d** Sections of ovaries of females exposed to 143.35 mg/L. Disorganization of the ooplasm of the oocytes at perinucleolar stage (*arrow* in **c**) and irregularities in the contour of the nuclear membrane (*arrows* in **d**) are observed. **e**, **f** Sections of ovaries of females exposed to 230.96 mg/L. The ovarian histological structure is altered. **e** Damage in the ooplasm of the oocytes at chromatin-nucleolar stage (*arrows*) and disorganization of ooplasm of growing oocytes (*asterisk*) are observed. **f** Hypertrophied follicle cells (*arrows*)

Effects on ovary histology

The ovaries of *G. atripinnis* (Fig. 1) and *C. encaustus* (Fig. 2) consisted of oocytes arranged in the wall of the ovary; the ovarian cavity was bounded by the internal ovarian epithelium. In *G. atripinnis*, oocytes at different stages of development (chromatin-nucleolar and perinucleolar stages) were simultaneously present, with high-quality ooplasm, cell membranes, and nuclei (Fig. 1a, b). The ooplasm and nucleus of *C. encaustus* oocytes in the perinucleolar stage were of high quality, as well as the follicle cell layer (Fig. 2a, b). The occurrence of atresia in the control groups was minimal, with one to two atretic oocytes per ovary.

Histology revealed that the ovaries of *G. atripinnis* females exposed to 2,4-D showed structural alterations versus the ovaries of females of the control group. According to the criteria defined by Braunbeck et al. (2010), which described changes related to potential endocrine disruption effects, we found two of the four primary diagnostic criteria in *G. atripinnis*. These were an increase in oocyte atresia and hypertrophy of follicle cells. At a dose of 143.35 mg/L, oocyte atresia was evidenced in the perinucleolar stage by disorganization of ooplasm (Fig. 1c) and by irregularities in the contour of the nuclear membrane



(Fig. 1d). At this dose, both diagnostic criteria were moderate changes (grade 3). Similarly, at a 2,4-D dose of 230.96 mg/L, the ovaries of exposed *G. atripinnis* females exhibited altered histological structure. The changes detected in the oocyte ooplasm included two signs of atresia: the ooplasm had a reduced volume in

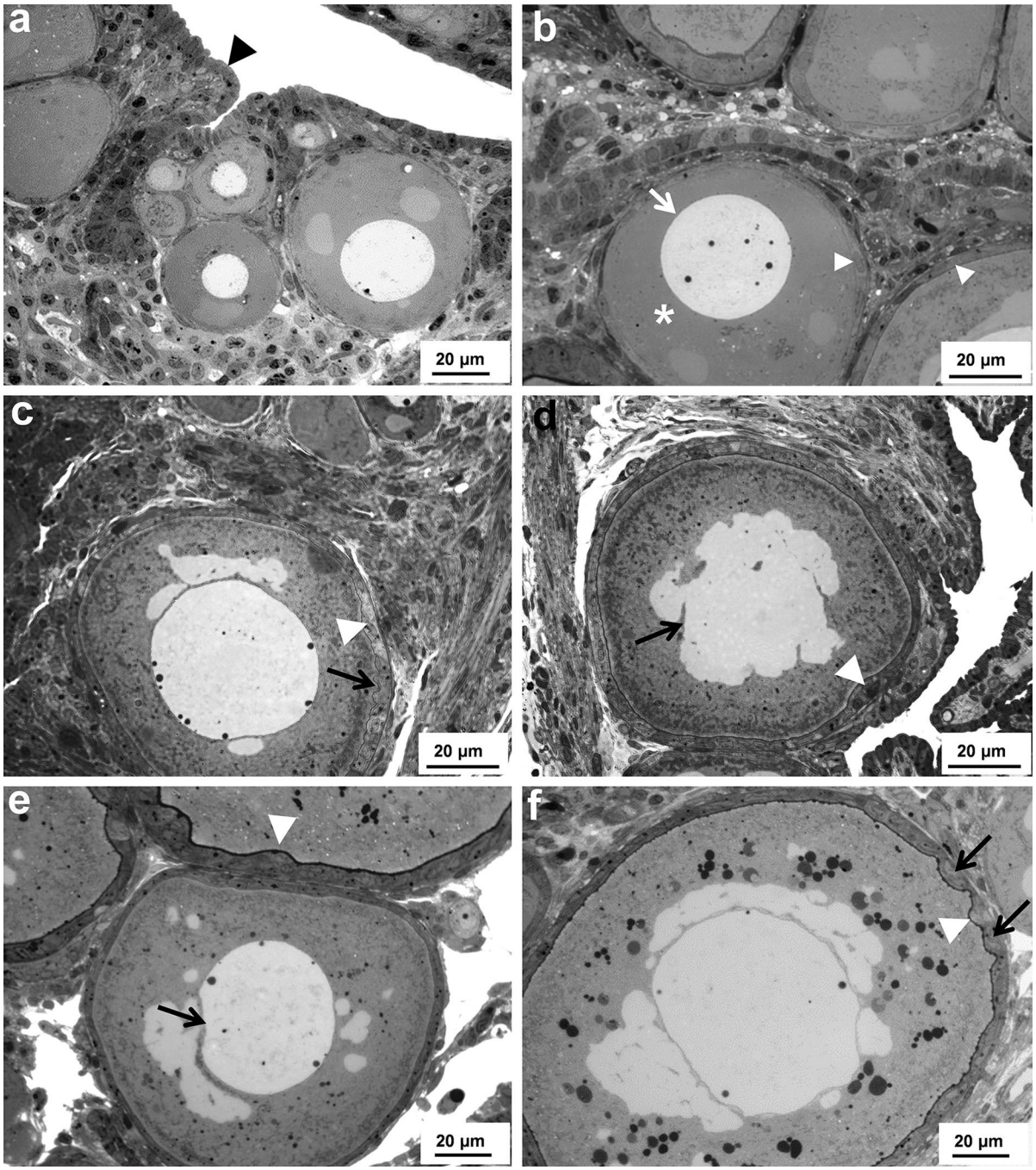


Fig. 2 Light microscopy of ovarian tissue of control and exposed females of *Chapalichthys encaustus*. **a**, **b** Sections of control ovary. **a** The ovarian cavity is bounded by the internal ovarian epithelium (*arrowhead*). **b** High-quality ooplasm (*asterisk*) and nuclei (*arrow*) of perinucleolar oocytes are observed, as well as intact follicle cell layer (*arrowheads*). **c**, **d** Sections of ovaries of females exposed to 94.06 mg/L. **c** Atretic oocytes at the perinucleolar stage. Oocyte membrane folding (*arrow*) and hypertrophy of some follicle cells enclosing these oocytes (*arrowhead*) are observed. **d** Oocyte at early vitellogenesis, alterations of the nuclei contour (*arrow*) and some hypertrophied follicular cells (*arrowhead*) are observed. **e**, **f** Sections of ovaries of females exposed to 133.72 mg/L. **e** Alterations of the nuclei contour of an atretic perinucleolar oocyte (*arrow*) and oocyte membrane folding of an early vitellogenic oocyte (*arrowhead*). **f** Oocyte membrane folding (*arrowhead*) and some hypertrophied follicular cells (*arrows*) in an early vitellogenic oocyte are observed

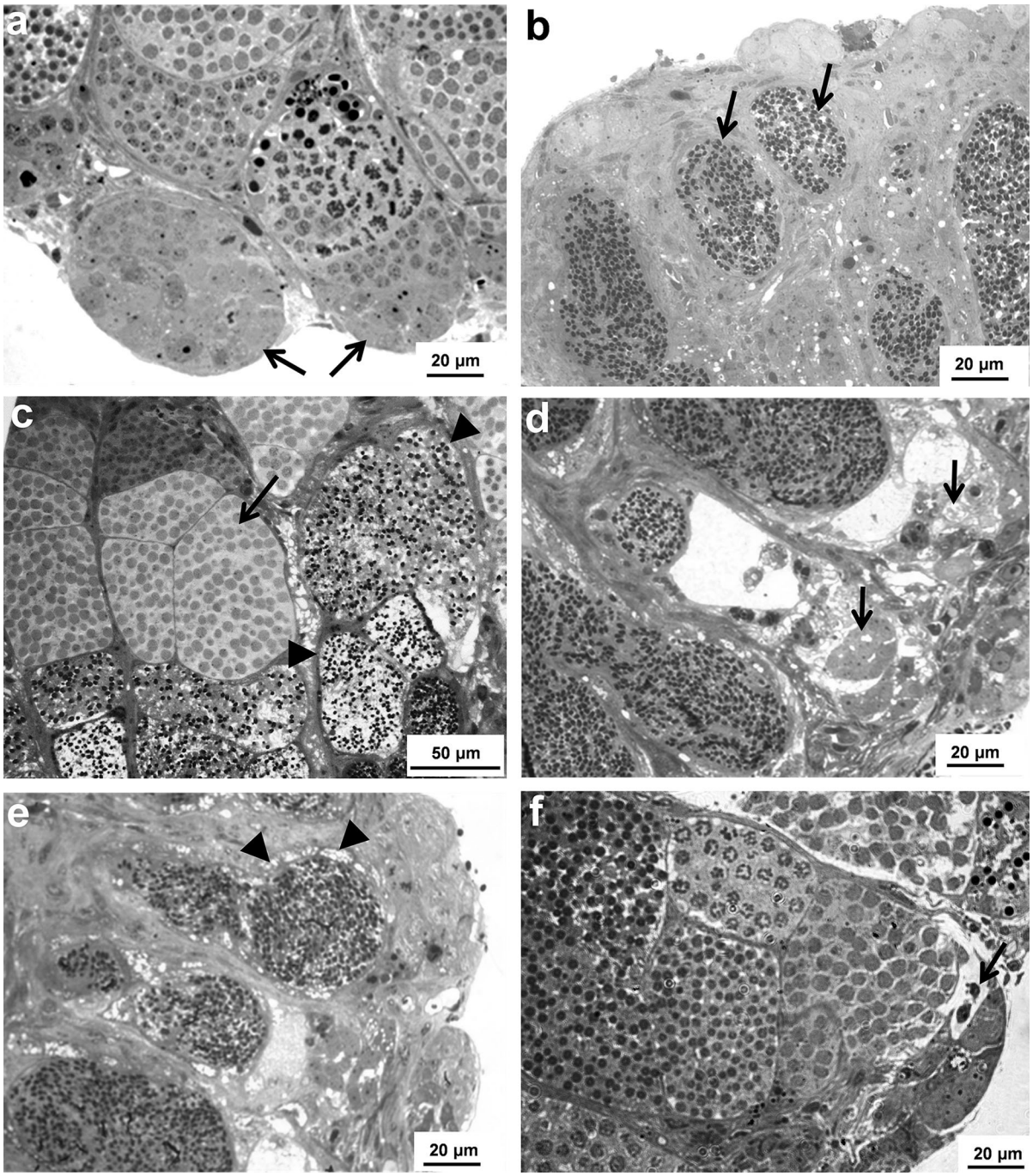


Fig. 3 Light microscopy of testicular tissue of control and exposed fish of *Goodea atripinnis* and *Chapalichthys encaustus*. **a**, **b** Sections of testis of control male of *G. atripinnis*. **a** Spermatogonial cysts (*arrows*). **b** Sperm cysts (*arrows*). **c** Spermatocytes cysts (*arrow*) and spermatids cysts (*arrowheads*) of control male of *C. encaustus*. **d, e** Sections of testis of *G. atripinnis* exposed to 230.96 mg/L. **d** Vacuolization of spermatogonia (*arrows*). **e** Alterations in the structure of sperm cysts (*arrowheads*). **f** Section of testis of *C. encaustus* exposed to 133.72 mg/L, alterations of the structure of spermatogonial cysts (*arrow*) are observed

the chromatin-nucleolar stage; and the ooplasm was disorganized in growing oocytes (Fig. 1e). We also observed follicle cell hypertrophy (Fig. 1f). At this dose, both diagnostic criteria were severe changes (grade 4). The histological structures of the ovaries were also altered in exposed female *C. encaustus*. We found two of the four primary diagnostic criteria defined by Braunbeck et al. (2010). These were an increase in oocyte atresia and follicle cell hypertrophy. At a dose of 94.06 mg/L 2,4-D, ovaries of exposed females showed signs of early atresia, evidenced by oocyte membrane folding (invaginations of the chorion) in perinucleolar oocytes. The follicular cell layer enclosing these oocytes showed hypertrophy (Fig. 2c). Early vitellogenic oocytes also showed irregular nuclear contours and hypertrophied follicular cells (Fig. 2d). At this dose, both changes were moderate (grade 3). Similarly, at a dose of 133.72 mg/L 2,4-D, the ovaries of exposed *C. encaustus* females showed an increase in oocyte atresia, evidenced by changes in the contours of nuclei in perinucleolar oocytes (Fig. 2e). Oocyte membrane folding and hypertrophied follicular cells were also observed in early vitellogenic oocytes (Fig. 2f). Both changes at this dose were severe (grade 4).

Effects on testis histology

The testes of *G. atripinnis* (Fig. 3a, b) and *C. encaustus* (Fig. 3c) consisted of cysts containing cells in various stages of spermatogenesis: spermatogonia, spermatocytes, spermatids and spermatozoa. We did not detect alterations in the testes of *G. atripinnis* or *C. encaustus* exposed to 2,4-D at doses of 143.35 and 94.06 mg/L, respectively. The Level of severity was classified as not remarkable.

In contrast, exposure of *G. atripinnis* males to 2,4-D at a dose of 230.96 mg/L led to alterations in the testis histology. Some spermatogonia located at the periphery of the testis showed vacuolization (Fig. 3d). This change is a feature of testicular degeneration, a primary diagnostic criterion. The level of severity for this dose was mild (grade 2). Cells forming the wall of sperm cysts (modified Sertoli cells) also showed vacuolization (Fig. 3e).

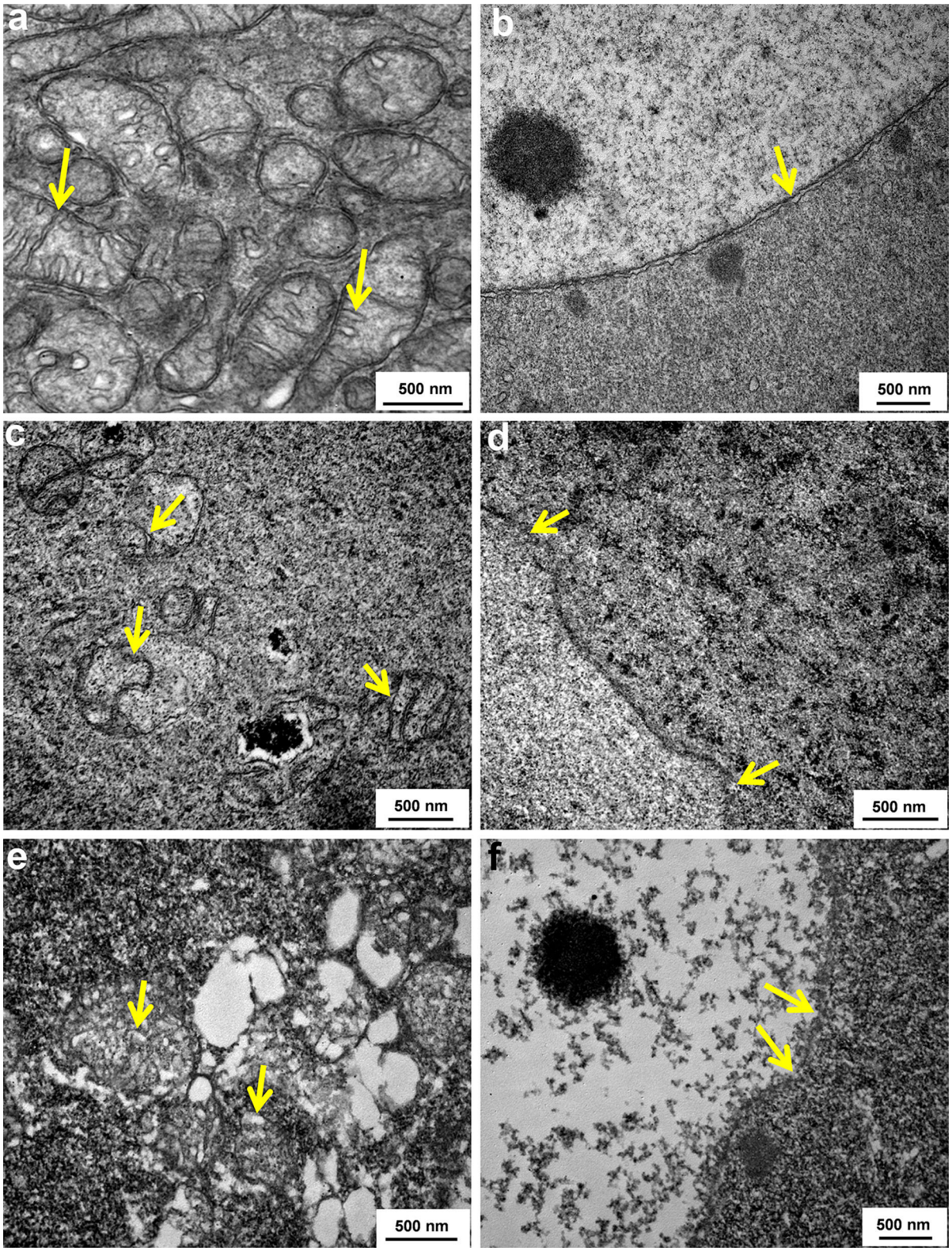
The testes of *C. encaustus* exposed to 2,4-D at a dose of 133.72 mg/L displayed changes in the structure of some spermatogonial cysts (Fig. 3f), while spermatids cysts appeared similar to those in control fish. The level of severity for this dose was classified as not remarkable, since we did not detect a change associated to a particular diagnostic criterion.

Fig. 4 Oocyte ultrastructure of control and exposed females of *Goodea atripinnis*. **a**, **b** Control ovary. **a** Mitochondria of an oocyte with intact cristae (*arrows*). **b** Intact nuclear membrane of an oocyte (*arrow*). **c**, **d** Ovary of females exposed to 143.35 mg/L. **c** Altered mitochondrial membranes of an oocyte (*arrows*). **d** Altered nuclear membrane of an oocyte (*arrows*). **e**, **f** Ovary of females exposed to 230.96 mg/L. **e** Altered mitochondrial membranes of an oocyte (*arrows*). **f** Loss of continuity of the nuclear membrane of an oocyte (*arrows*)

Effects on ovary ultrastructure

At the ultrastructural level, ovaries from control females of *G. atripinnis* (Fig. 4a, b) and *C. encaustus* (Fig. 5a, b) contained oocytes with intact mitochondrial cristae and nuclear membranes. Oocytes from female *G. atripinnis* exposed to 2,4-D at a dose of 143.35 mg/L displayed altered mitochondrial membranes (Fig. 4c) and nuclear membranes (Fig. 4d). Similarly, in the ovaries of female *G. atripinnis* exposed to 2,4-D at a dose of 230.96 mg/L, we detected damaged mitochondrial membranes in oocytes (Fig. 4e) and a loss of continuity in the nuclear membranes of oocytes (Fig. 4f). Exposure of *C. encaustus* to 94.06 mg/L resulted in changes in the structure of the mitochondrial membranes of oocytes (Fig. 5c) and in the continuity of the nuclear membrane of oocytes (Fig. 5d). Similarly, exposure of *C. encaustus* to 133.72 mg/L of 2,4-D led to loss of integrity in the mitochondrial membranes of oocytes (Fig. 5e) and changes in the continuity of the nuclear membranes of oocytes (Fig. 5f).

Control ovaries from *G. atripinnis* and *C. encaustus* contained intact follicular cells at the ultrastructural level (Fig. 6a, b). *G. atripinnis* females exposed to 2,4-D at a dose of 143.35 mg/L displayed a loss of integrity of the cytoplasm of the surrounding follicular cells (Fig. 6c) and exposure of *G. atripinnis* to 230.96 mg/L resulted in follicular-cell dystrophy (Fig. 6d). In the ovaries of *C. encaustus* females exposed to 94.06 mg/L, changes in the structure of the mitochondrial membranes of follicular cells were observed (Fig. 6e). Similarly, exposure of *C. encaustus* to 133.72 mg/L of 2,4-D led to altered mitochondrial membranes of follicular cells (Fig. 6f).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Effects on testis ultrastructure

In testes from control *G. atripinnis* (Fig. 7a) and *C. encaustus* (Fig. 7b), mitochondria in spermatids were intact. When *G. atripinnis* males were exposed to 2,4-D at a dose of 143.35 mg/L, testes displayed alterations in the mitochondria of spermatids (Fig. 7c). Similarly, *G. atripinnis* males exposed to 2,4-D at a dose of 230.96 mg/L, exhibited changes in the mitochondria of spermatids (Fig. 7d). Alterations in mitochondrial cristae in spermatids were evident in *C. encaustus* at a dose of 94.06 mg/L (Fig. 7e) and at a dose of 133.72 mg/L (Fig. 7f).

Histopathological index

Four histopathological indexes were evaluated for *G. atripinnis* and *C. encaustus*. Two were indexes for females, including increased oocyte atresia (Iatr) and follicle cell hypertrophy (Ifh); and two were indexes for males, including plasma alterations (Ipa) and structural alterations (Ist). According to the classification of Bernet et al. (1999), vacuolization of spermatogonia could be included in the plasma alterations for males. For females, the highest index values were observed in Iatr; *G. atripinnis* displayed an Iatr of 72 (at 230.96 mg/L of 2,4-D), and *C. encaustus* displayed an Iatr of 67.5 (at 133.72 mg/L of 2,4-D). For males, the highest index values were 10.5 for Ipa in *G. atripinnis* (at 230.96 mg/L of 2,4-D) and 7.5 for Ist in *C. encaustus* (at 133.72 mg/L of 2,4-D). Significant differences ($p \leq 0.5$) were detected between females and males and between different doses in *G. atripinnis* (Fig. 8a) and in *C. encaustus* (Fig. 8b). No significant differences were found between females of the two species. However, significant differences were observed ($p \leq 0.5$) between males of the two species.

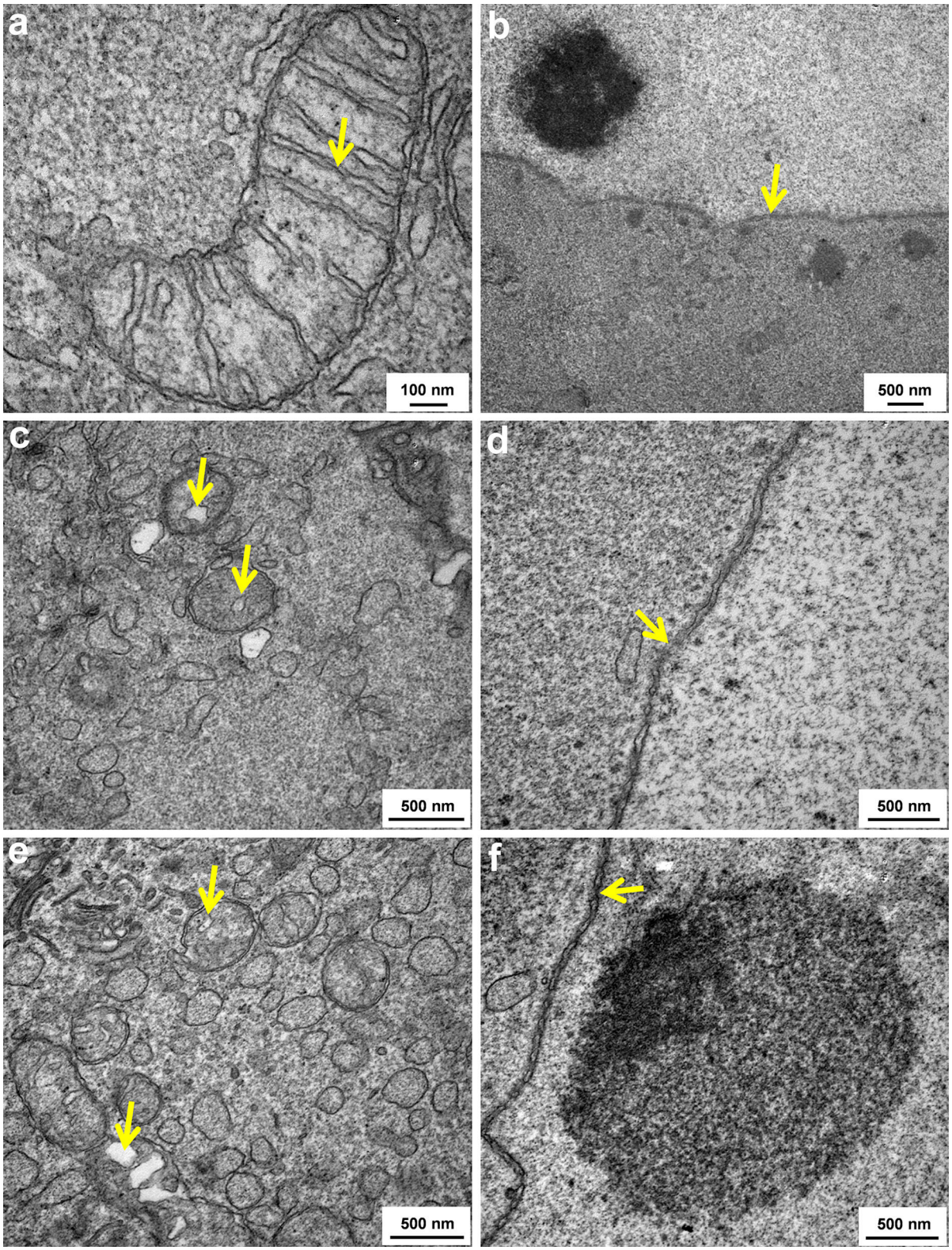
Discussion

The gonads of *G. atripinnis* and *C. encaustus* exposed to 2,4-D showed alterations in histological structure and ultrastructure. Some of these alterations matched primary diagnostic criteria that have been related to potential endocrine disruption effects. In the ovaries, alterations included an increase in oocyte atresia, follicle cell hypertrophy, and changes in oocyte mitochondrial and nuclear membranes. In the testes, alterations included vacuolization of spermatogonia, changes in the structural integrity of the spermatogonial cyst, and changes in the mitochondrial membranes of spermatids. Therefore, several of the alterations we found could be related to 2,4-D disruption of endocrine functions, including oocyte atresia, follicle cell hypertrophy, and spermatogonia vacuolization.

Fig. 5 Oocyte ultrastructure of control and exposed females of *Chapalichthys encaustus*. **a**, **b** Control ovary. **a** Intact mitochondrial cristae of an oocyte (*arrow*). **b** Intact nuclear membrane of an oocyte (*arrow*). **c**, **d** Ovaries of females exposed to 94.06 mg/L. **c** Altered mitochondrial membranes of an oocyte (*arrows*). **d** Loss of continuity in the nuclear membrane of an oocyte (*arrow*). **e**, **f** Ovaries of females exposed to 133.72 mg/L. **e** Altered mitochondrial membranes of an oocyte (*arrows*). **f** Changes in the continuity of the nuclear membrane of an oocyte (*arrow*)

Lc_{50}

Despite the widespread use of pesticides in Mexico (Valdez Salas et al. 2000; Sánchez-Guerra et al. 2011), to date, no data have been published on the LC_{50} of 2,4-D for the Goodeinae. The data obtained in this study establish the baseline toxicity of 2,4-D, which is important when evaluating and comparing the effects observed in the field and in the laboratory. The present study determined toxicity data (LC_{50}) for viviparous fish of the Goodeinae subfamily for the first time using a commercial formulation of 2,4-D. Comparison of the LC_{50} values obtained in this study with data reported for other species indicates that *C. encaustus* and *G. atripinnis* can be classified as moderately sensitive to 2,4-D. Comparison of these LC_{50} values indicated that *C. encaustus* is more sensitive than *G. atripinnis* to the 2,4-D herbicide. For viviparous teleosts there is a study of *P. reticulata* (Rehwoldt et al. 1977). Those authors used pure 2,4-D and found a LC_{50} (96 h) of 70.7 mg/L. For oviparous teleosts like *Geophagus brasiliensis* (Cichlidae), a LC_{50} (96 h) of 15.16 mg/L was found using pure 2,4-D, indicating high sensitivity of this species to 2,4-D (Barbieri 2009). Moreover, for rainbow trout (*Oncorhynchus mykiss*), the LC_{50} (96 h) for a 2,4-D commercial formulation (ESTHER'H) is 494 mg/L (Fairchild et al. 2009). Therefore, commercial formulations of 2,4-D is an important factor affecting its toxicity to fish.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The concentrations of this herbicide reported in the environment are lower than the LC_{50} for 2,4-D determined here. For example, 0.05 ppm 2,4-D was detected in Lake Chapala (Mexico) in the rainy season (Arévalo-Hernández et al. 2011), and a mean concentration of 0.28 μg/L (maximum value 10.4 μg/L) was reported for California (USA), also in the rainy season (Ensminger et al. 2013). Although environmental concentrations of contaminants are lower than the concentrations used in the laboratory, a constant exposure, even at doses considered sublethal, can exert long-term effects on the health of organisms. Since 2,4-D is still used as a pesticide in agriculture, it is constantly discharged into bodies of water, especially during the rainy season; runoff increases its concentration (Arévalo-Hernández et al. 2011). We evaluated the effect of 2,4-D in isolation. Under natural conditions, species are exposed to a mixture of pesticides (or other xenobiotics) that may interact and produce additive, synergistic or antagonistic effects (Payne et al. 2000).

Effects on histology and ultrastructure

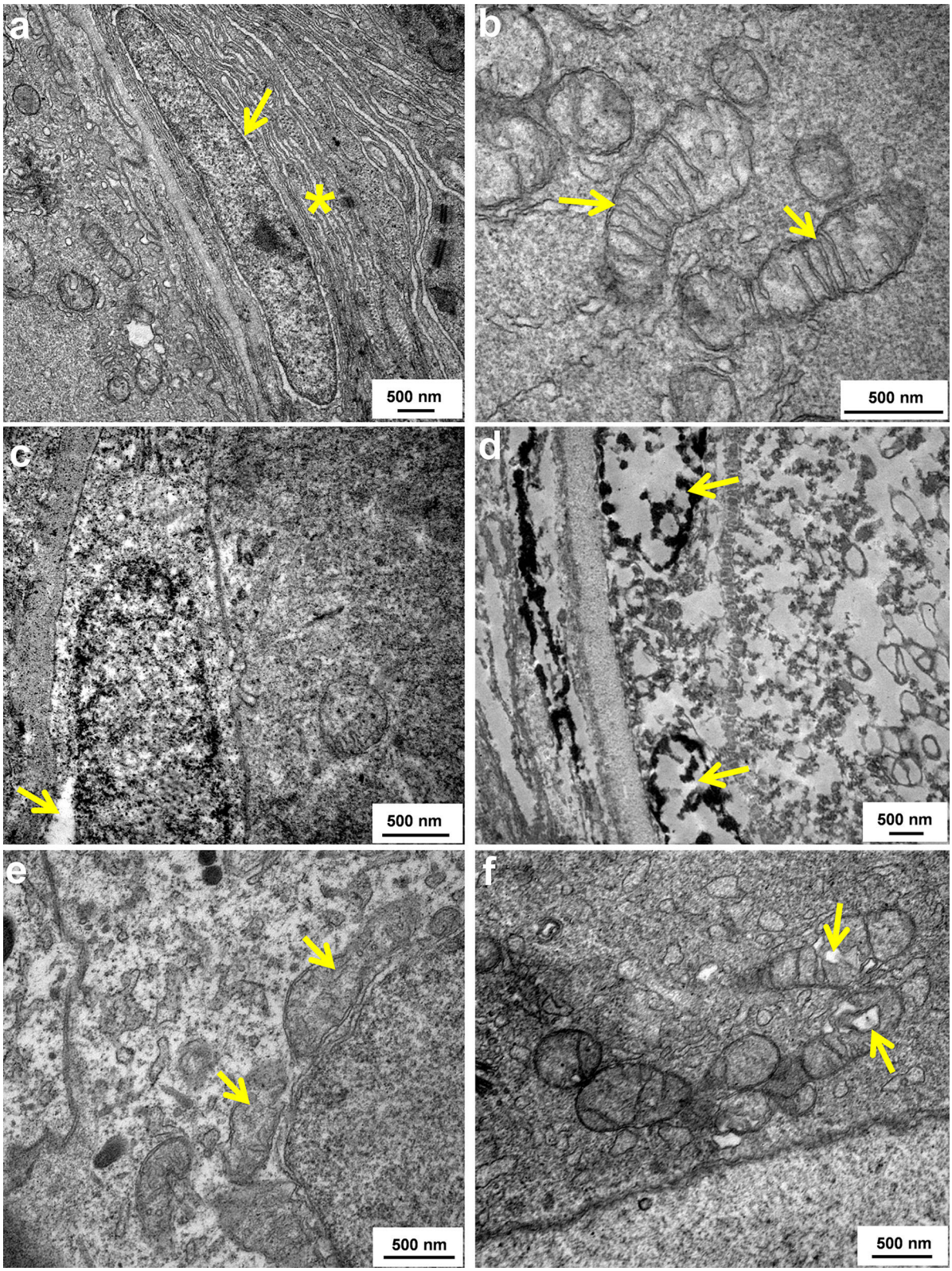
We observed a relationship between exposure to this herbicide and alterations in gonad histology and ultrastructure in both studied species. Ovarian histology was affected in *C. encaustus* and *G. atripinnis* at both employed doses. The primary diagnostic characteristics that could be related to endocrine disruption were oocyte atresia and follicle cell hypertrophy. Both characteristics were increased in the ovaries of exposed females compared to the ovaries of unexposed control females. According to the description reported by Lambert (1970), the observed changes in oocytes could be classified as changes in phase α of atresia, because they showed early features of degradation, which coincided with the short time of exposure (96 h).

No changes in testis histology were detected for *G. atripinnis* exposed at 143.35 mg/L or *C. encaustus* exposed at 94.06 mg/L. According to the Braunbeck et al. (2010) classification of severity, no remarkable changes were detected at these doses (the lowest employed in this study). Thus, the diagnostic criteria related to endocrine disruption indicated that 2,4-D exposure did not affect the testes at low doses. However, at higher doses, the histology showed structural changes in the testes. After exposure to 230.96 mg/L for *G. atripinnis* and 133.72 mg/L for *C. encaustus*, alterations in the spermatogenic cysts were observed. Nevertheless, the only primary diagnostic characteristic that could be related to endocrine disruption in the testes of exposed males was vacuolization of spermatogonia, which was observed in *G. atripinnis* at the highest dose employed (230.96 mg/L). Alterations in ovarian follicles and spermatogenic cells may reflect damage to the process of gametogenesis and may affect the number of gametes produced, which may reduce the reproductive capacity of individuals over the long term (Arellano-Aguilar and Macías-García 2008).

Fig. 6 Follicle cell ultrastructure of control and exposed females of *Goodea atripinnis* and *Chapalichthys encaustus*. **a** Intact cytoplasm (*asterisk*) and nucleus of a follicular cell (*arrow*) of control ovary of *G. atripinnis*. **b** Intact mitochondria of a follicular cell of control ovary of *C. encaustus* (*arrows*). **c**, **d** Changes in exposed *G. atripinnis* females. Loss of integrity of the cytoplasm of a follicular cell (*arrow*) of a female exposed to 143.35 mg/L. **c** Dystrophy of follicular cells (*arrows*) of a female exposed to 230.96 mg/L. **e**, **f** Alterations in exposed *C. encaustus* females. **e** Mitochondria of a follicular cell with altered cristae (*arrows*) of a female exposed to 94.06 mg/L. **f** Loss of integrity of mitochondrial membranes of a follicular cell (*arrows*) of a female exposed to 133.72 mg/L

We observed ultrastructural alterations in the gonads of both *C. encaustus* and *G. atripinnis* at the doses employed here. In the ovary, changes occurred in the mitochondrial membranes of oocytes and follicular cells, as well as in the nuclear membrane of the oocyte. In the testes, alterations were evident in the mitochondrial membranes of spermatids. In the catfish *Clarias batrachus*, changes in the histology and ultrastructure of gonads following exposure to 2,4-D were previously reported (Ateeq et al. 2006). Those authors found that exposure to 2,4-D prompted changes in the mitochondria in the ovary and slight effects on mitochondria of the testes. On the contrary, we found alterations in mitochondria of ovaries as well as testes of both studied species.

Although no effects on testis histology were observed for *C. encaustus* and *G. atripinnis* at 2,4-D doses of 94.06 and 143.35 mg/L, respectively, testis ultrastructure was affected, indicating that changes to mitochondrial membranes constitute an early warning damage. Chronic exposure may cause this early damage to scale from the subcellular level to the levels of tissues and organs. In fish, the bioaccumulation of pesticides has been reported mainly in liver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and muscle (Van der Oost et al. 2003). 2,4-D is known to accumulate in liver specimens from *G. atripinnis* inhabiting Lake Chapala (Arévalo-Hernández et al. 2011). The authors of that study suggested that damage to the genetic material of liver cells may have been caused by exposure to 2,4-D. Other organs may also be targeted during the accumulation of contaminants. The present study uncovered the effects of exposure on the gonads, suggesting that 2,4-D reaches these organs. Accumulation of organochlorine pesticides has been reported in gonads of wild individuals of the oviparous fish *Odontesthes bonariensis* (Barni et al. 2014).

The matrotrophic viviparous nature of these species indicates that the presence of contaminants such as 2,4-D (and its possible accumulation in the ovary) may affect embryonic development when substances pass from the mother to the developing embryos and are absorbed by the trophotaeniae or other embryonic structures. Since 2,4-D potentially disrupts endocrine activity (Mnif et al. 2011), it may affect sexual differentiation, gonadal development, and offspring sex ratio, affecting the reproductive health of individuals and populations.

Previous studies have described 2,4-D as a potential ED (Mnif et al. 2011). In rainbow trout, 2,4-D increased the levels of plasma vitellogenin, which suggested estrogenic activity (Xie et al. 2005). In *Xenopus* sp., 2,4-D inhibited oocyte maturation (Stebbins-Boaz et al. 2004; LaChapelle et al. 2007). In contrast, recent studies by Coady et al. (2013, 2014) did not find endocrine disruption activity with 2,4-D tested in vitro. According to those authors, the reduced fecundity found in *Pimephales promelas* fish at the highest 2,4-D concentration tested was most likely due to a generalized stress response in the fish, rather than a specific endocrine action of 2,4-D.

According to the diagnostic criteria for histological changes related to endocrine disruption, some of the changes observed in this study (increased oocyte atresia, follicle cell hypertrophy, and vacuolization of spermatogonia) could be related to endocrine disruption by 2,4-D exposure. However, other observed alterations probably arose due to a general toxic effect of the high doses tested. Taking into account the diagnostic effects observed here, we conclude that the viviparous Goodeid species, *C. encaustus* and *G. atripinnis*, may be used as models of endocrine disruption.

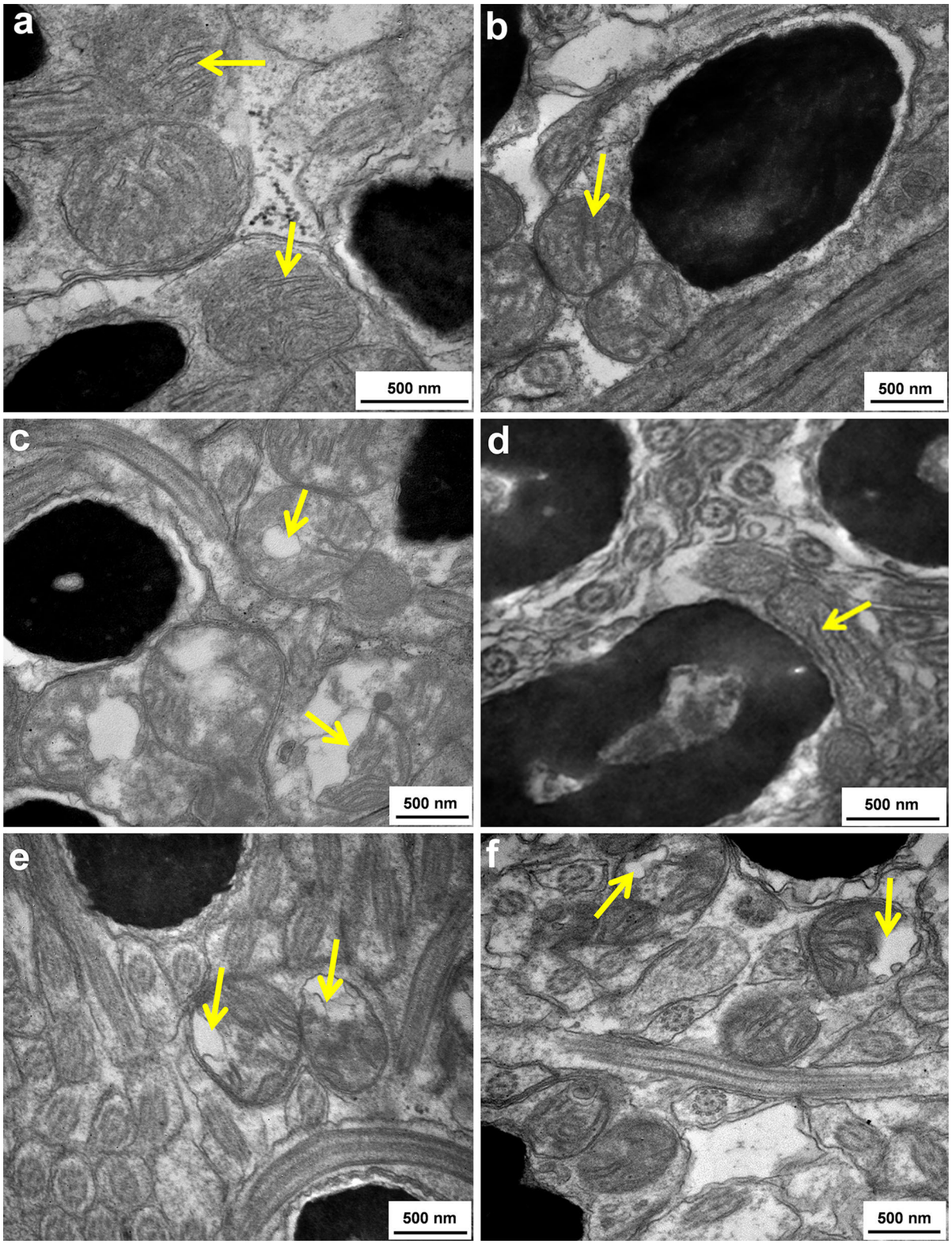
Fig. 7 Testicular ultrastructure of control and exposed fish of *Goodea atripinnis* and *Chapalichthys encaustus*. **a**, **b** Control testis with intact mitochondrial membranes of spermatids of *G. atripinnis* (*arrows* in **a**) and *C. encaustus* (*arrow* in **b**). **c**, **d** Testes of *G. atripinnis* males exposed to 2,4-D. Altered mitochondrial cristae of spermatids of males exposed to 143.35 mg/L (*arrows* in **c**) and of males exposed to 230.96 mg/L (*arrow* in **d**). **e**, **f** Testes of *C. encaustus* males exposed to 2,4-D. Alterations in mitochondrial cristae of spermatids of males exposed to 94.06 mg/L (*arrows* in **e**) and of males exposed to 133.72 mg/L (*arrows* in **f**)

Viviparous teleosts with intraluminal gestation as models of endocrine disruption

Viviparous teleost with IG which display matrotrophy are appropriate models to understand aspects of endocrine disruption in viviparous vertebrate reproduction, such as prenatal programming, maternal-embryonic relationship, postnatal health and reproductive health (Edwards et al. 2010). Viviparous fish can be remarkable models in these studies by their reproductive characteristics, with advantages over oviparous species. For example, viviparous offspring are protected by their mother, enabling evaluation of the effects of pollutants on progeny size, sex ratio, size, weight, and developmental malformation. This information would allow comparative studies of organisms from contaminated sites, from reference sites, and even from the laboratory. However, we should take into account that some characteristics of fish could be disadvantages in endocrine disruption studies, for example, chromosome duplication (Storz et al. 2013; Glasauer and Neuhauss 2014) and the complex steroid hormone system, which is a feature common to all fish (Borg 1994).

The effects of EDs on the reproduction of viviparous teleosts with IG have been most extensively studied in *Z. viviparus* in the laboratory and in the field, as well as at several levels of organization. The Goodeidae family has been less extensively studied at all organizational levels, with few studies conducted at the physiological and individual levels and none on the effects of EDs during gestation. In this context, viviparous Goodeidae species are appropriate models of endocrine disruption because they are matrotrophic and because the embryos have trophotaeniae.

Due to the great diversity in the reproductive strategies of viviparous teleosts, progeny from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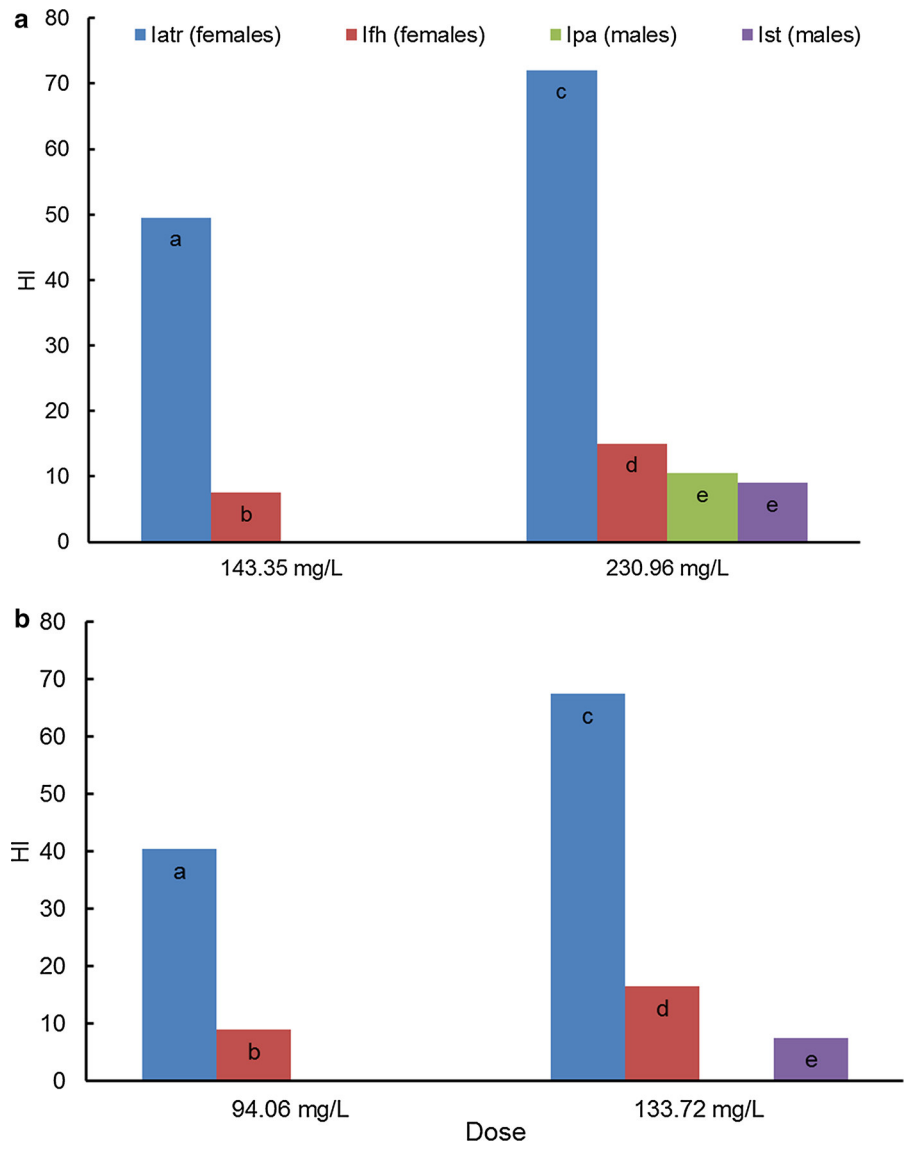

Fig. 8 Histopathological index (HI). **a** *Goodea atripinnis*. **b** *Chapalichthys encaustus.* Oocyte atresia index (*Iatr*); follicle cell hypertrophy index (*Ifh*); plasma alterations index (*Ipa*); structural alterations index (*Ist*). Different letters represent significant differences ($p \leq 0.5$)

different species may vary in their responses to EDs. For example, in Goodeinae and Zoarcidae species, embryos are in contact with the ovarian fluid and, for this reason, the transfer of pesticides from the mother to the embryos could be greater than in Poeciliidae species, in which embryos develop separately inside each follicle. Nutritional patterns can also influence responses to endocrine disruption. For example, Goodeinae and Zoarcidae species exhibit a high level of matrotrophy, implying a greater dependence on maternal support, while most Poeciliidae species display various grades of lecitotrophy, with greater dependence from yolk reserves. It is therefore of great interest to compare the effects of EDs on progeny from species with different sites of gestation (intraluminal vs. intrafollicular) and with different nutritional patterns (lecitotrophic vs. matrotrophic).

Endocrine disruption in viviparous fish is a new field of research. However, in recent years, biological knowledge of model species has grown. Despite the physiological differences between fish and other vertebrates, some common characteristics, like the formation of placental analogues, may facilitate applying the knowledge gained in viviparous fish to

other viviparous vertebrates. In this regard, matrotrophic viviparous teleosts with IG could be particularly informative.

Gaps in research and future research directions

There are many gaps in our knowledge of endocrine disruption in viviparous teleosts with IG. Most studies have been conducted at the levels of cells and individuals, with few investigations addressing the levels of genes and populations. In this regard, it is of interest to determine gene sequences and patterns of gene expression in order to assess changes in the expression of genes that may be indicators of impaired reproduction, such as genes encoding steroidogenic hormones, steroid hormone receptors, and genes that function in sexual determination and sexual differentiation like *sox9*, *dmrt1*, *amh, foxl2*, *cyp19* and *sf-1*(Guerrero-Estévez and Moreno-Mendoza 2010; Leet et al. 2011; Shen and Wang 2014).

Similarly, studying contaminant exposure at the levels of populations and ecosystems is crucial to understanding the effects of pollutants on the sustainability of viviparous populations. In this context, pesticide concentrations should be measured in the field at various times of the year and compared with concentrations that cause damage (both acute and chronic) in laboratory tests. For example, to assess whether environmental pollution underlies changes in the reproductive success of species in their natural environments, the development of methods or markers to determine the effects of exposure on reproduction in situ would be very useful.

Information at various levels of organization comes mainly from separate investigations of EDs conducted under laboratory conditions; field studies are limited, and have focused on the population level for particular species. Thus, assessment of the effects of potential EDs in natural environments must include: studies in which fish are exposed to water from sites contaminated with EDs, in order to assess the alterations on reproduction (at different levels of biological organization); characterization of pesticides with potential endocrine disruption action from polluted sites; and exposure of individuals to ecologically relevant concentrations of EDs alone and in mixtures.

Contributions from various levels of biological organization

Endocrine disruption must be assessed from a holistic perspective that accounts for different levels of organization in order to understand the mechanisms by which EDs impact reproduction. Molecular studies provide insight into the intracellular mechanisms by which EDs act. At the cellular level, some responses could be quantified (e.g. via sperm count), as could damage to organelles. At the level of the individual, gonadosomatic index may be an indicator of pesticide effect. At this level, pathological conditions such as the development of ovotestes or atrophied gonads could explain consequences on the reproductive fitness of individuals in natural populations. The physiological level is crucial because it reflects processes at the molecular and cellular levels; investigations at this level enable the formulation of inferences about effects on individuals. For example, alterations in the time and level of expression of steroid hormones can lead to changes in the sexual maturity of individuals (precocious or delayed), with possible consequences on reproduction. Importantly, data obtained at the level of the individual provide insight into potential effects on populations. For example, alterations of secondary sexual characteristics can affect the reproductive fitness of the entire population. Likewise, changes in sexual determination and sexual differentiation in individuals can be reflected in populations as deviations in sex ratio, possibly leading to population decline over the long term. Finally, effects detected at the population level enable predictions of possible repercussions on several populations in a community and their effects on ecosystems.

Conclusions

Viviparous fish may serve as models for endocrine disruption studies in viviparous vertebrates. These fish offer some advantages to field studies; for example, the offspring are protected by the mother. However, some of their characteristics, common to all teleosts, may constitute limitations in endocrine disruption studies; for example, chromosome duplication and a complex steroid hormone system.

Endocrine disruption in viviparous fish is a new field of research. It would be informative to compare the effects of EDs on progeny from species with different sites of gestation (intraluminal vs. intrafollicular) and with different nutritional patterns (lecitotrophy vs. matrotrophy). The diagnostic histological changes observed in this study (increased atresia, follicle cell hypertrophy, and vacuolization of spermatogonia) could be related to 2,4-D exposure, and possibly, to an endocrine disruption activity. The oocytes showed early features of atresia, which coincided with the short time of exposure to 2,4-D (96 h). However, other histological changes could be related to stress, induced in response to the high doses employed.

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