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Effects of cypermethrin chronic exposure and water temperature on survival, growth, sex differentiation, and gonadal developmental stages of *Odontesthes bonariensis* (Teleostei)

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ABSTRACT

This study assessed the effects of cypermethrin and temperature on the survival, growth, sex differentiation, and gonadal development of *Odontesthes bonariensis*, a gonochoristic teleost with a strong thermolabile sex differentiation. Two complementary trials were conducted. In the first trial, newly hatched larvae were exposed during six weeks to 0 or 0.1 μg L^{-1} of cypermethrin at 17, 22, and 29 °C. In the second trial, larvae were exposed at 22 °C to 0, 0.1, or 0.125 μg L^{-1} of cypermethrin, or 100 μg L^{-1} of the non-steroidal antiestrogen tamoxifen. Survival and growth of fish were affected by cypermethrin exposure, water temperature, and the combination of both factors. The survival rate decreased at higher temperatures and cypermethrin concentrations, but the insecticide lethality was inversely related to temperature. Growth was lower at 17 °C than at 22 or 29 °C, and was significantly increased by cypermethrin exposure. As already described for this species, all females or all males were obtained at 17 or 29 °C, respectively, and neither cypermethrin nor tamoxifen exposure caused changes in sex ratios. Slight changes in gonadal development were induced only by temperature. Finally, results showed that the *in vitro* antiestrogenic effect reported for cypermethrin had no *in vivo* effects on the sex ratio, the gonadal development, or the germ cell production of *O. bonariensis*, even at concentrations that affected the growth and survival of the fish.

1. Introduction

Cypermethrin is a synthetic pyrethroid broadly used for residential, commercial, industrial, and agricultural pest control originally associated with cotton crops (WHO, 1989). In recent years it has also been adopted for pest control in soybean culture (Carriquiriborde et al., 2007), a crop that is increasing in developing countries and currently exceeds 26 million hectares in South America alone (James, 2007). Residues of cypermethrin have been widely detected in water and sediment samples from streams and rivers draining major agricultural districts (Marino and Ronco, 2005).

It is well known that cypermethrin is extremely toxic to fish and aquatic arthropods under laboratory conditions (Bradbury and Coats, 1989), and it is also recognized that its toxicity is reduced under field conditions in water bodies with abundant particulate material (Hill, 1989). For example, a 12-fold increase of the LC50 at 96 h was observed for the fish *Cnesterodon decemmaculatus* exposed to cypermethrin in stream-water when compared to laboratory waters. Furthermore, no lethal effects were observed after insecticide exposure under field-use conditions (Carriquiriborde et al., 2007). However, concern exists about the possible sublethal effects induced by this pyrethroid, particularly taking into account that cypermethrin spraying events mainly occur during the breeding season of many fish species.

Cypermethrin has also been reported to exhibit *in vitro* antiestrogenic activity (Tyler et al., 2000). Estradiol is a key hormone in ovarian differentiation in many fish species (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002), therefore the potential capacity of cypermethrin to act as an endocrine disruptor *in vivo* should be taken into consideration. Moreover, a recent study reported that *in vivo* waterborne exposures to cypermethrin affected gonadotrophic cells, gonads, and concentration of steroids in the plasma of the catfish, *Heteropneustes fossilis* (Singh and Singh, 2008).

The selected experimental model, pejerrey (*Odontesthes bonariensis*), is a native inland-water fish from the southern sector of the "del Plata Basin" that usually inhabits shallow ponds within important agricultural districts of Argentina, Uruguay, and Brazil (Somoza et al., 2008). This fish is a gonochoristic teleost with weak or possibly

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absent genetic control of the sex differentiation process. Rather, sexual differentiation is strongly dependent on the water temperature during the first weeks after hatch, a temperature-sensitive period (Strüssmann et al., 1996a). In this species, the sex ratio is influenced over a broad range of temperatures; for example, all-female and all-male populations result when the fish are kept at 17 and 29 °C, respectively. Mixed-sex groups are produced at intermediate temperatures during the temperature-sensitive period (Strüssmann et al., 1996a, 1997). However, during this period, the male/female ratio can be modified by the administration of exogenous estradiol (Strüssmann et al., 1996b). This fact not only points out the relevance of estradiol during the sex differentiation process in pejerrey, but also shows the potential impact that endocrine disruptors may have on this process.

Temperature is also an important factor modifying the toxicity of contaminants (Sprague, 1995), and it has been specifically shown that the toxicity of pyrethroids to fish can depend on the water temperature (Haya, 1989). Therefore, the combined effects of pesticide exposure and temperature could be relevant for the survival and reproductive success of this species.

Within this conceptual framework, the aim of the present study was to evaluate the combined effects of cypermethrin exposure and water temperature on survival rate, growth, and sex ratio of pejerrey larvae during the temperature-sensitive window under laboratory conditions.

2. Materials and methods

Two complementary experiments were conducted. In the first trial, groups of 30 fish were subjected to six different treatments resulting from the combination of two waterborne concentrations of cypermethrin (0 and 0.1 $\mu g\,L^{-1}$) and three rearing temperatures (17, 22, and 29 °C). In the second trial, groups of 50 fish were subjected only to three different treatments resulting from three waterborne concentrations of cypermethrin (0.00, 0.100, and 0.125 $\mu g\,L^{-1}$) at 22 °C. Additionally, in this second trial, a fourth group was included, exposing 50 fish to tamoxifen (100 $\mu g\,L^{-1}$), a non-steroideal anti-estrogen with a demonstrated masculinizing action on other fish species (Kitano et al., 2007). Both trials were conducted in triplicate, and fish were subjected to these different treatments from hatching to week 6. Then, the fish were kept in clean water at 22 °C after week 6 until week 18 or 12 in the first or second trial, and sacrificed for histological analysis of the gonads.

Newly hatched *O. bonariensis* were obtained from the IIB-INTECH broodstock and transported in polyethylene bags to the CIMA aquatic facilities. Once arrived, the fish were gradually acclimated to the test water (tap water, dechlorinated and filtered trough activated charcoal) and rearing temperatures for 48 h.

Forty-eight hours post-hatch larvae were used for experiments. Fish were exposed in 2-L glass vessels containing the different test solutions, kept in thermo-regulated baths (17 and 29 °C) or at room temperature (22 °C). The photoperiod was set at 16 h light and 8 h dark. Cypermethrin was added to the water dissolved in ethanol, and the same ethanol concentration was added to all control groups (0.0100% or 0.0125% in trial 1 or 2, respectively). Tamoxifen working solutions were prepared immediately before use and dissolved in deionized water. Static renewal was performed replacing 100% of the test solution volume every 48 h.

Fish were daily fed with 5 $\mu L\,fish^{-1}$ of concentrated newly hatched brine shrimp *nauplii* (*Artemia sp.*) at the start of the experiment. This amount of food was increased by 5 $\mu L\,fish^{-1}$ every week until the end of the experiments. Each day, dead fish were removed from test jars, survival rate was recorded every 48 h and the standard length (SL) measured to the nearest 0.1 mm for growth assessment at week 6.

For histological gonadal identification, the samples were taken at week 12 or 18, depending on the experiment. The fish were anesthetized to death with an overdose of benzocaine (ethyl 4-aminobenzoate), cut opened, fixed in Bouin's fluid overnight at 4 °C, and then preserved in 70% alcohol. They were then dehydrated in an ascending alcohol series, embedded in paraffin, and cross-sectioned at 6 μm. The histological preparations were then stained with hematoxylin–eosin and examined under a microscope. The stages of gonadal development were classified following the method of Strüssmann et al. (1996b) and grouped according to the characteristics that follow.

2.1. Ovarian developmental stages

Stage I: Characterized by the presence of large germ cells and clusters of somatic cells at the ventral and hilar edges. A main blood vessel is observed in all individuals (Fig. 1a).
Stage II: The ovarian primordial presented outgrowths of the somatic cells in the ventral and hilar edges. Large germ cysts are present (Fig. 1b).
Stage III: The somatic outgrowths are fused delimiting the ovarian cavity. Perinucleolus oocytes are present (Fig. 1c).

2.2. Testicular developmental stages

Stage I: Presumptive testis has a pear-like shape with a low germ/somatic cell ratio (Fig. 1d).
Stage II: The main sperm duct and germ cells are present (Fig. 1e).
Stage III: The main sperm duct and spermatocytes cysts are present (Fig. 1f).

In the second trial, the mean number of germ cells, large germ cell cysts, and perinucleolar oocytes per histological section was analyzed from images taken of the most developed region of the left and right gonad of 15 fish per treatment. Each analyzed slide contained 20 transverse body sections taken 100 μm apart from each other.

Once a week, from each test chamber, one liter of water was collected just after a renewal, and before of the following replacement, for the determination of the concentration of cypermethrin and tamoxifen. Cypermethrin concentration was analyzed by GC-EC according to Marino and Ronco (2005). Tamoxifen in water was measured by HPLC-MS. Briefly, test water was filtered through a 0.45 μm syringe filter and 20 μL directly injected to an Agilent 1100 HPLC system connected with a quadrupole mass spectrometer (Agilent MSD-VL) equipped with an ESI interface. Chromatographic separation was performed on a reversed-phase Gemini C18 analytical column (150 mm × 4.6 mm, 5 μm particle diameter) under isocratic conditions using 40% acetonitrile and 60% ammonium acetate buffer. MS analysis was performed in the SIM mode (*m/z* 372.5) with the ESI interface operating in positive ion mode. The limit of detection of the method was 0.9 $\mu g\,L^{-1}$.

Technical grade cypermethrin of 91% purity was kindly provided by GLEBA S.A (La Plata, Argentina) and tamoxifen was purchased at Droguería Saporiti (Buenos Aires, Argentina). All solvents used were pesticide analysis grade.

Statistical analyses were assessed by 2-way and 1-way ANOVA for the first and second trial, respectively. Comparison of means for the first trial was performed using the *post hoc* Tukey's HSD (Honestly Significant Difference) test. Survival data were arc-sine square root transformed, and homoscedasticity and normality assessed by the Bartlett and Shapiro Wilk's tests, respectively (USEPA, 2002). Sex ratios, gonadal development stages, and germ cell frequencies among control and exposed groups were compared using the χ^2-test. In all cases, a significant value of $P < 0.05$ was established.

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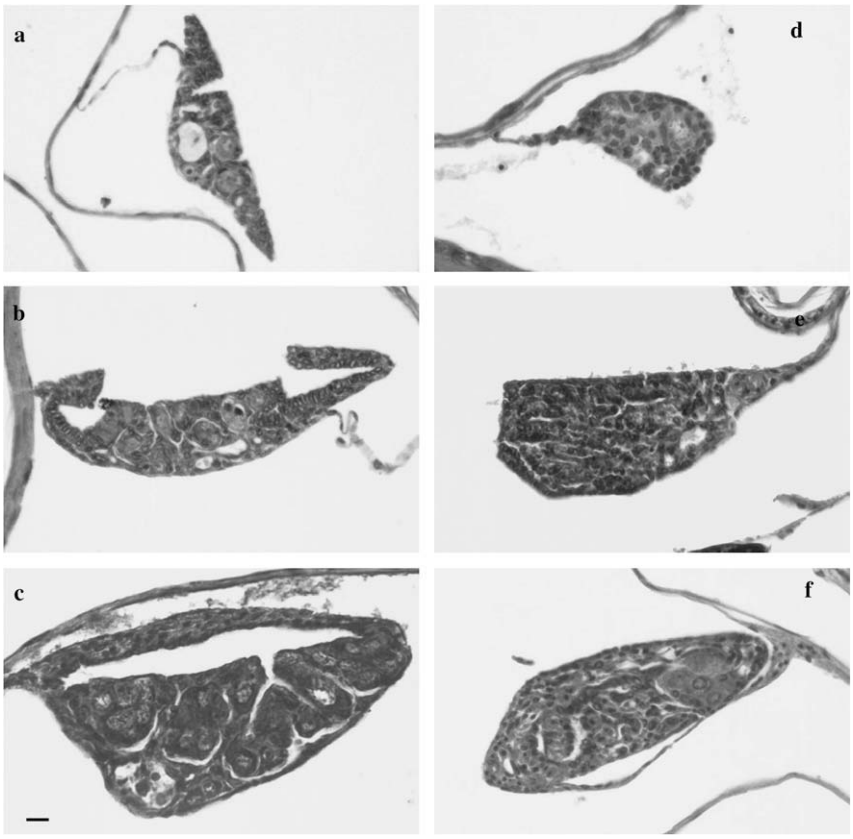

Fig. 1. Gonadal developmental stages in larvae of pejerrey. Ovary stages: (a) I; (b) II; (c) III. Testis stages: (d) I; (e) II; (f) III. Bar equal to 10 μm.

3. Results

Mean concentration of cypermethrin measured in test solutions from the two experiments were within 97.6 ± 3.8% (n = 12) of the nominal values immediately after renewal; but, this value fell to 31.2 ± 4.5% (n = 12) after 48 h. No significant differences in cypermethrin concentration were observed among tested temperatures during the first trial. During the second trial, the mean tamoxifen concentration values were 85.0 ± 4.1% (n = 6) of the nominal value immediately after water renewal, but they dropped to 9.4 ± 8.6% (n = 6) after 48 h.

The mean survival of pejerrey larvae observed during the first and second experiments is shown in Figs. 2 and 3. During the first experiment, significant effects on the survival were induced by temperature, cypermethrin, and by their interaction. Major mortalities occurred during the first two weeks and ceased almost completely after the third week after hatching. The survival rates in control groups were inversely related to temperature, and values for control groups at 22 and 29 °C were, after six weeks, 2- and 7-fold lower than at 17 °C, respectively (Fig. 2b). Cypermethrin caused a significant reduction in survival rate in fish exposed at 17 and 22 °C, but not at 29 °C when compared to non-exposed fish (Fig. 2b). The reduction of the survival rate attributable to cypermethrin at the end of the exposure period was 90.5% and 49.3% at 17 and 22 °C, respectively, whereas no significant effects were observed at 29 °C (Fig. 2b).

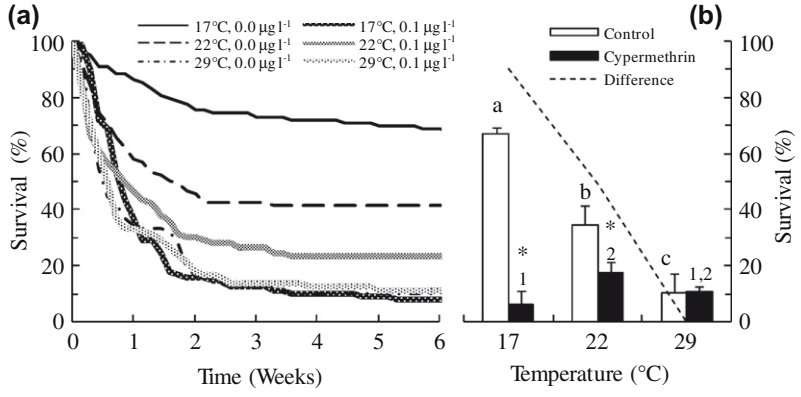

Fig. 2. Survival of pejerrey larvae exposed to 0 or 0.1 μg L^{-1} of cypermethrin during the first six weeks after hatching at 17, 22, or 29 °C. (a) Time-dependent survival curves for the different treatments; (b) comparison of the fish survival at the end of the exposure period for the different treatments. Different letters indicate statistically significant differences (P < 0.05) among non-exposed groups at different temperatures. Different numbers indicate statistically significant differences (P < 0.05) among exposed groups at different temperatures. Asterisk indicates statistically significant differences (P < 0.05) between exposed and non-exposed groups at the same temperature.

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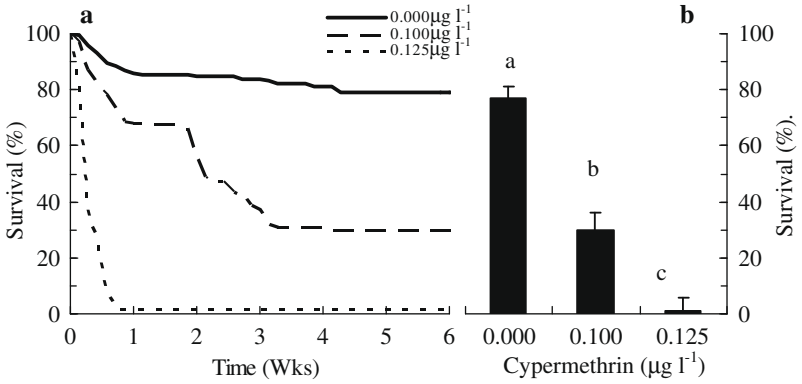

Fig. 3. Survival of pejerrey larvae exposed to 0, 0.100, and 0.125 µg L^{-1} of cypermethrin during the first six weeks after hatching at 22 °C. (a) Time-dependent survival curves for the different treatments; (b) comparison of the fish survival at the end of the exposure period for the different treatments. Different letters indicate statistically significant differences ($P < 0.05$) among different treatments.

Table 1
Growth response of *O. bonariensis* to the different assessed treatments during the first and the second trial.

Trial	Cypermethrin concentration (µg L^{-1})	Temperature (°C)					
		17		22		29	
		Mean SL (mm)	SE	Mean SL (mm)	SE	Mean SL (mm)	SE
1st	0.0	14.8	0.24^{1a}	17.8	0.36^{2a}	16.5	0.77^{2a}
	0.1	16.4	0.63^{1a}	18.6	0.42^{2a}	19.0	0.49^{2a}
2nd	0.0	–	–	17.3	0.47^{a}	–	–
	0.1	–	–	18.7	0.08^{b}	–	–

Different numbers or letters indicate statistical differences ($P < 0.05$; $N = 3$) in fish length among temperatures or cypermethrin concentration, respectively. SL: standard length.

A significant reduction of the survival rate of fish was also induced by cypermethrin in the second experiment, following a clear concentration-dependent pattern (Fig. 3a and b). The reduction in the survival rate at the end of the exposure period was 61.4% and 98.8% in fish exposed to 0.100 and 0.125 µg cypermethrin L^{-1}, respectively, compared with non-exposed fish. Although the time-dependent mortality pattern was similar between both trials, the survival rate was approximately 10% greater in the second trial, possibly showing batch differences. In addition, a significant reduction of fish survival with respect to the control group was observed in the fish exposed to tamoxifen (36.0 ± 5.8%).

The effects of the different treatments on fish growth, expressed as standard length (SL) at the end of week 6, during the first and second trials are shown in Table 1. In the 0.0125 µg cypermethrin L^{-1} treatment, growth rate was not evaluated because the small number of surviving fish did not allow a statistical analysis of the data. During the first trial, fish growth was significantly affected by both temperature and cypermethrin exposure as determined by ANOVA. Although *post hoc* comparison showed that SL was only significantly lower in fish at 17 °C with respect to those at 22 and 29 °C, the SL means of fish exposed to cypermethrin were always higher with respect to unexposed control fish at all studied temperatures. The same effects were observed during the second trial. No significant correlation was found between survival and growth, in the first (slope 0.029; $\beta = -0.403$; $n = 18$) or the second trial (slope 0.021; $\beta = -0.601$; $n = 6$).

The sex ratios obtained during the first trial are shown in Fig. 4a. There were no differences between cypermethrin-exposed and non-exposed fish, either at female (17 °C)- or male (29 °C)-producing temperatures. As expected, 100% females and males, respectively, were obtained at each temperature in the control groups. In the 22 °C groups, although control fish were all female, two of the three replicates treated with cypermethrin showed 26.6% and 23.3% of males. However, these differences were not statistically significant.

No differences were observed in the degree of the ovarian development when control and cypermethrin-exposed fish were compared at 17 and 22 °C (Fig. 4b). At 17 °C, all females presented ovaries in stage III, whereas at 22 °C, a small percentage of stage I ovaries (2.4–3.0%) was also observed. No stage II ovaries were found in any of these experimental groups. The distribution of the testicular developmental stages obtained at 22 and 29 °C are shown in Fig. 4c. At 22 °C, males were only observed in the group exposed to cypermethrin; all stages of testicular development were found with a prevalence of stage II. At 29 °C, the testicular maturation stages were comparable between exposed and control groups, and characterized by a prevalence of stage III testis (88.9% and 69.5% in controls and exposed fish, respectively), with a smaller proportion in stage II (11.1% both groups). The proportion of fish exposed to cypermethrin with testes in stage I was 19.4%. However, the distribution frequency was not significantly different from that observed in the control group ($P = 0.765$).

During the second trial, differences in sex ratios among controls and fish exposed to cypermethrin or tamoxifen were not observed. In this case, 100% of the experimental fish were females (Fig. 5a). In addition, the gonadal developmental stages in cypermethrin- and tamoxifen-exposed animals were similar to those observed in control fish, showing a prevalence of stage II ovaries (Fig. 5b). No significant differences were observed in the mean number of germ cells ($P = 0.864$), large germ cell cysts ($P = 0.963$), or perinucleolus oocytes per histological section ($P = 0.964$) (Fig. 5c).

4. Discussion

The rapid reduction of waterborne cypermethrin concentration observed during the experiments was consistent with previously

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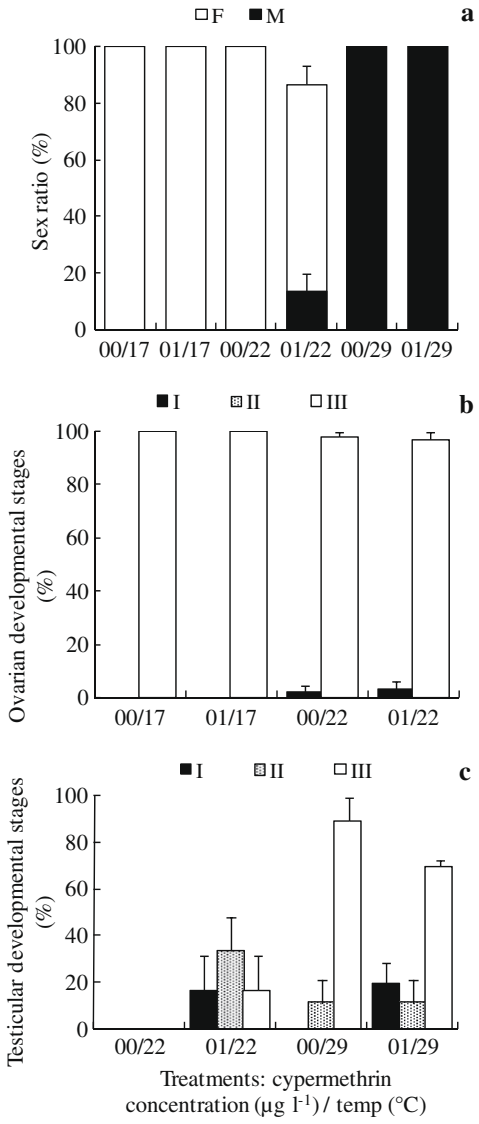

Fig. 4. Sex ratio and gonadal development stages observed in *O. bonaerensis* during the first trial. (a) Sex ratio; (b) ovarian developmental stages; (c) testicular developmental stages. F: Females; M: males. I, II, and III: gonadal developmental stages (see Fig. 1).

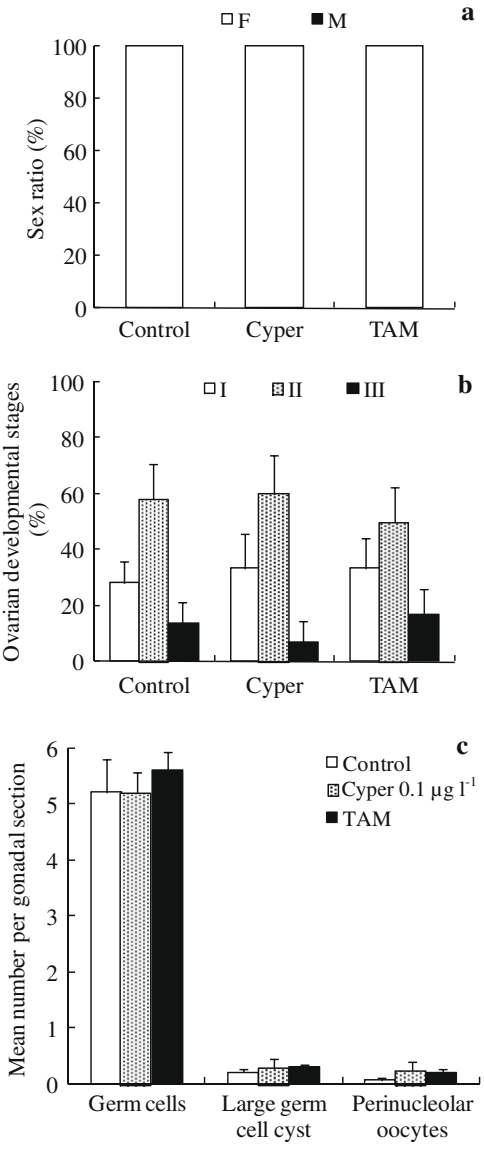

Fig. 5. Sex ratio, gonadal development stages, and number of different cell types observed during the second trial. (a) Sex ratio; (b) ovarian developmental stages; (c) number of germ cells, large germ cell cysts, and perinucleolar oocytes. F: Females; M: males. I, II, and III: gonadal developmental stages (see Fig. 1).

published results using static exposure systems (Edwards et al., 1987; Lutnicka et al., 1999). This reduction was mainly attributed to the bioaccumulation and metabolism of the insecticide (Edwards et al., 1987).

The spontaneous mortality rate in control fish obtained in this study appeared to be high; although it was similar with previously reported data for early life stages of pejerrey (Miranda et al., 2006). In addition, the variability of the spontaneous mortality rates observed among experiments and its increase at greater temperatures were similar to the results published by Strüssmann et al. (1997). High spontaneous mortality rates at high temperatures have been also reported for early life stages of other fish species (Baras et al., 2001).

Despite the normally high spontaneous mortality rate of pejerrey larvae, the effects of cypermethrin on fish survival were clear, showing a negative correlation with water temperature. This observation is consistent with reported data for pyrethroids because they exhibit a negative temperature coefficient of toxicity to fish (Haya, 1989).

Growth of fish is a physiological process strongly dependent on water temperature, existing optimal temperatures for different species, and developmental stages (Brett, 1979). When temperatures become sub- or super-optimal, the effect is a reduction in growth (Jobling, 1997). In the present study, fish length showed an increase from 17 to 22 °C, with no changes at 29 °C. This is consistent with the growth-optimal temperatures reported for this species (Strüssmann and Yasuda, 2005).

In the present study, a marginal but significant increase in the mean length of fish was observed in all groups exposed to cypermethrin. Previously reported effects of pyrethroids on fish growth vary among studies, depending on the insecticide, the species, and the experimental conditions. For example, growth rate of *Cyprinodon variegatus* in early life stages was shown to be reduced when exposed to the pyrethroid AC222 during 28 d, but it was shown to be increased when exposed to permethrin and fenvalerate under the same experimental conditions (Hansen et al., 1983). Fenvalerate has also been demonstrated to have a bimodal effect on *Atherinops affinis* growth; being stimulatory at 0.31 µg L^{-1} but inhibitory at

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0.62 μg L^{-1} (Goodman et al., 1992). However, no effects on growth were reported for esfenvalerate on *Lepomis macrochirus* exposed during 90 d to concentrations up to 0.05 μg L^{-1} (Little et al., 1993). Culture density also influences fish growth (Schram et al., 2006). In addition, it has been observed that growth rates of leopard frogs larvae are indirectly affected by esfenvalerate as consequence of changes in culture density (Materna et al., 1995). However, it seemed not to be the case for the present experiments, since no relationship was found between survival and growth rate. Although the mechanism of action of cypermethrin on growth is not known in pejerrey, the observed increase in growth rates could be explained by its ability to increase the spontaneous, calcium-dependent, release of dopamine in the brain (Soderlund et al., 2002). Dopamine is a well known GH-secretagogue in teleosts (Peter and Marchant, 1995; Björnsson et al., 2002; Canosa et al., 2007).

Another process controlled by water temperature in pejerrey is sex determination. In the present study, 100% females and 100% males were consistently obtained in the control groups at 17 and 29 °C, respectively. Variable sex ratios can be obtained at intermediate temperatures (Strüssmann et al., 1997); in this case, 100% females were obtained in the two experiments conducted as control groups and reared at 22 °C, which is also consistent with previous data by Strüssmann et al. (1997) and Karube et al. (2007).

It has also been shown that estradiol is crucial for gonadal sex differentiation in teleosts (Guiguen et al., 1999; Kitano et al., 2007). In pejerrey for instance, oral administration of 50 mg kg^{-1} estradiol from days 28 to 105 after hatching produced all females (Strüssmann et al., 1996b), and gonadal aromatase expression is up-regulated at the female-producing temperature (Karube et al., 2007; Fernandino et al., 2008). The oral administration of an antiestrogenic compound, tamoxifen, caused 100% masculinization of Japanese flounder (*Paralichthys olivaceus*) raised at a female-producing temperature during the sex differentiation period (Kitano et al., 2007). If cypermethrin can act *in vivo* as an antiestrogen substance, as reported *in vitro* (Tyler et al., 2000), an action on the pejerrey gonadal sex differentiation process could logically be expected. However, waterborne exposure of pejerrey larvae to cypermethrin during sexual differentiation did not affect sex proportion in fish raised at either 17 or 29 °C. The "masculinizing effects" of cypermethrin seen during the first trial at an intermediate temperature (22 °C) could not be later verified and may have represented the normal variability of sex ratios at intermediate temperatures (Strüssmann et al., 1997).

In the present conditions, the lack of effects of cypermethrin on the sex differentiation process of pejerrey was also supported by the absence of changes in the gonadal development stages between exposed and non-exposed fish. The differences in the gonadal development stages were clearly distinguished between the first (mainly stage III) and the second trial (mainly stage II) due to the differences in the sampling time (weeks 18 and 12, respectively).

Tamoxifen, a non-steroidal antiestrogen, blocks the interaction of estradiol with the estrogen receptors (Sutherland et al., 1977), and it has been reported to have a potency of about 1000 times greater than cypermethrin (Tyler et al., 2000). In addition, oral administration of tamoxifen was reported to induce masculinization in the Japanese flounder (Kitano et al., 2007). In the present study, although the concentration of tamoxifen was high enough to induce a significant mortality, it did not induce masculinization in pejerrey. This lack of effect of tamoxifen on sex ratio has also been reported in rainbow trout and tilapia using oral administration (Guiguen et al., 1999). The present results would suggest that either waterborne exposure to tamoxifen is not capable of reaching target organs in pejerrey or sex differentiation in pejerrey is not sensitive to antiestrogens. Other methods of tamoxifen administration and different antiestrogenic drugs should be tested to verify this hypothesis. In this context, the lack of effect of cypermethrin should be interpreted in a similar way.

From an ecological point of view, previous studies showed that cypermethrin toxicity under field-use conditions is drastically reduced by particulate and dissolved organic matter and direct lethal effects of the insecticide are rarely expected on wild fish (Carriquiriborde et al., 2007). Then, the present results indicate that long-term exposure to relatively high concentrations of cypermethrin causes mortality during the first days (acute lethal response), but surviving or tolerant fish showed only slight sublethal effects on growth, with no evident alterations on the sex differentiation process or gonadal development. However, the compromise of reproduction during adulthood should be taken into consideration and tested.

5. Conclusions

This study confirms the high toxicity (lethality) of cypermethrin to fish under laboratory conditions, and shows a clear inverse relationship between toxicity of cypermethrin and water temperature. Conversely, only subtle effects on larval growth were observed, and *in vitro* antiestrogenic effects reported for cypermethrin were not able to affect, *in vivo*, sex ratio, gonadal developmental, or germ cell production in *O. bonariensis* chronically exposed to relatively high concentrations of the insecticide.

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