Ecotoxicology and Environmental Safety 140 (2017) 116–122

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Histopathological effects of chlorpyrifos on the gills, hepatopancreas and gonads of the freshwater crab *Zilchiopsis collastinensis*. Persistent effects after exposure

C.L. Negro[a,b,*], P. Collins[a,b]

[a] Instituto Nacional de Limnología (CONICET-UNL), Paraje El Pozo s/n, 3000 Santa Fe, Argentina
[b] Escuela Superior de Sanidad, Facultad de Bioquímica y Ciencias Biológicas, UNL Paraje El Pozo s/n, 3000 Santa Fe, Argentina

ARTICLE INFO

Keywords:
Chlorpyrifos exposure
Crustaceans
Histopathologies
Persistent effects

ABSTRACT

Sublethal effects of the pesticide chlorpyrifos were evaluated in the crab *Zilchiopsis collastinensis* (Decapoda, Trichodactylidae). Crabs were exposed to high concentrations of chlorpyrifos at the beginning of the experiment and controlled dilution, under natural light and temperature conditions. A control and three concentrations (22.4, 41.25 and 61.4 μg chlorpyrifos L^{-1}) were evaluated in triplicate. Nine crabs per concentration and day were used. The gills, hepatopancreas and ovaries were sampled before pesticide exposure (day 0) and 8, 15 and 22 days later, when concentrations were diluted and below the detection limits. The histopathological effects and their variations in time were observed and quantified. In gills, hyperplasias were observed in several cases, mainly in crabs exposed to chlorpyrifos. The number of collapsed lamellae and the number of affected lamellae quickly increased in exposed crabs, as effects were observed on day 8 and remained until day 22. In hepatopancreas there was an increase in the number of F and B -cells and affected tubules, especially after 22 days of exposure ($p < 0.05$). In ovaries, there were no effects on gonadosomatic indexes or oocyte volume, but there was a significant increase in the atretic oocyte proportion related to pesticide exposure ($p < 0.05$). The histopathological effects on the gills, hepatopancreas and ovaries were observed after exposure and persist even after dilution, and might be related to earlier exposures. Thus, these histopathological effects might be used as pesticide biomarkers even after the pesticide is not detected by chemical methods.

1. Introduction

The increasing use of pesticides in agricultural activities is considered a major problem worldwide. After their application in crop fields, these compounds migrate to aquatic environments mainly by drift and runoff (Ernst et al., 1990; Marino and Ronco, 2005).

Chlorpyrifos is an organophosphate lipophilic pesticide (K_{ow} of 4.7) widely used in soy crops (Mugni et al., 2016). It is able to partition directly between the water and the exposed organism through the skin, during feeding and respiration (Tomlin, 1998; Newman and Unger, 2003).

Crabs are a common part of the biota of freshwater ecosystems, and those that inhabit near agricultural areas are periodically exposed to different pesticide concentrations (Collins et al., 2007). All toxicants effects begin by interacting with biomolecules. Their effects then cascade through the biochemical, subcellular, cellular and tissue levels, and eventually individual, population, community and ecosystem levels (Newman and Unger, 2003). The histopathological effects produced in different tissues of non-target organisms are usually used to biomonitor polluted areas and environmental conditions in aquaculture systems (Vogt, 1987; Mortimer, 2000; Varó, 2000). The use of histopathological biomarkers in biomonitoring has many advantages. The histological effects are consequences of biochemical mechanics that provide interpretative power about effects to individuals they integrate damage done at the molecular level (Hinton and Laurén, 1990). These biomarkers can be used as an early warning system for potential effects at the level of the individual and, sometimes, at the population level. They provide a cost-effective way to verify the effects produced by toxicant exposure (Newman and Unger, 2003).

The gills of aquatic animals are usually used to assess pollution effects, as they are the first organ which comes in contact with aquatic pollution. Also, they are highly vulnerable to toxic chemicals, mainly because their large surface area facilitates the interaction and their absorption. The absorption through gills is rapid and therefore the toxic response in gills is also rapid (Pandey et al., 2008).

In crustaceans the hepatopancreas is a dynamic organ, whose

* Correspondence to: Laboratorio de Macrocrustáceos. Instituto Nacional de Limnología (CONICET-UNL), Ciudad Universitaria, Paraje El Pozo s/n. Santa Fe, Santa Fe, Argentina.
E-mail address: leonegro82@hotmail.com (C.L. Negro).

http://dx.doi.org/10.1016/j.ecoenv.2017.02.030
Received 8 November 2016; Received in revised form 17 February 2017; Accepted 21 February 2017
0147-6513/ © 2017 Elsevier Inc. All rights reserved.

activities are related mainly to digestive functions, but also to detoxification and even gonadal development processes, as it is responsible for the production of vitellogenins, the precursors of egg yolk (Icely and Nott, 1992; Vogt, 2002). During secondary vitellogenesis, the lipids stored in the hepatopancreas are processed into lipoproteins and transported via the hemolymph to the oocytes (Harrison, 1990; Lubzens et al., 1995; Rodríguez et al., 2000).

Deleterious effects at the tissue level require a longer time of exposure than the effects at lower biological organization levels, as in the case of molecular level. The histopathological effects related to the exposure to sublethal concentrations require several days of exposure to be observed (Newman and Unger, 2003; Sreeram and Menon, 2005; Abad-Rosales et al., 2010; Wu et al., 2008; Negro, 2015). In natural scenarios the concentration of pesticides in water peaks in relation to the pesticides applications and/or rain events. After this peak concentration there is often a natural dilution (Ernst et al., 1990; Jergentz et al., 2005). So, several days after the inputs, when pesticide concentrations are already diluted, the histopathological effects on crabs might continue to be observed.

In general, the histopathological effects of pesticides are evaluated in constant exposure systems; where biocide concentrations are relatively stable (Reddy et al., 1983; Rodríguez et al., 1994; Bhavan and Geraldine, 2000, 2009). However, to our knowledge, there are few records of histopathological effects observed after pesticide concentrations decreases (Negro, 2015).

In the present study we used *Zilchiopsis collastinensis* (Decapoda: Trichodactylidae), a common crab of freshwater ecosystems related to the middle Paraná River, with one reproduction event in the year. In female crabs, the gonad gradually develops from winter to summer, when spawning occurs (Collins et al., 2007; Senkman et al., 2015). It is an active predator and detritus feeder and an important food source for fish, reptiles, birds and mammals, even human, with a central position in both the aquatic and terrestrial food webs, playing a key role both in matter and energy exchange in riparian zones, since it connects terrestrial and aquatic environments (Collins et al., 2007).

The aims of this work were to simulate a pollution scenario with a single contamination pulse of chlorpyrifos in a system with controlled dilution and to observe the histopathological effects produced in the gills, hepatopancreas and ovaries of *Zilchiopsis collastinensis* after the pesticide concentration decreased below the detection limit, as a way to observe effects that remain after the exposure.

2. Material and methods

2.1. Animal collection and acclimation conditions

Adult female *Zilchiopsis collastinensis* crabs were collected on the Paraná River floodplain (31°30′S, 60°41′W; Santa Fe, Argentina) in late winter. The crabs were acclimated for 14 days in natural light and temperature conditions. One hundred and forty-four intermolt crabs were placed in twelve 300 L aquaria filled with dechlorinated water. The water was renewed at a rate of 20 L per day in a continuous flow system and the excess water was drained by overflow. The aquaria contained plastic shelters to simulate the crab burrows. The mean (± SD) carapace width of the crabs used was 48.77 (± 3.75) mm. Additionally, the largest individual was not more than 1.5 times larger than the smallest individual, a criterion that has been proposed for fish assays (Reish and Oshida, 1987). The aquaria were placed in an open greenhouse covered with shade cloth, to avoid the overheating caused by direct sun exposure. The crabs were fed fresh fish muscle *ad libitum*. Food was supplied once in the evening, and the leftovers were removed early the next morning.

2.2. Bioassays

After the acclimation period, each aquarium received a single dose of commercial grade pesticide (Clorpi Ciagro®; Red Surcos S. A., Argentina) containing 48% chlorpyrifos. The commercial product was diluted in dechlorinated water, added to each aquarium and gently homogenized throughout the tank. Three initial concentrations (C1: 20; C2: 40; C3: 80 µg chlorpyrifos L^{-1}), which were about 1/8, 1/4 and 1/2 of the LC_{50-96h} were used (Negro et al., 2014). These concentrations were applied in a system with a high initial concentration followed by dilution with pesticide-free dechlorinated water, simulating the dilution process of aquatic ecosystems (Fig. 1). The dilution rate was 20 L per day in a continuous flow system and the excess water was drained by overflow, as before the pesticide application. A control group was subjected to the same conditions as the exposed groups, but without the addition of pesticides. Three replicates of each treatment were performed. Food was supplied to the crabs in the same manner in which it was supplied during the acclimation period. Dissolved oxygen, pH and conductivity were measured three times a week before feeding. The water temperature was recorded twice a day.

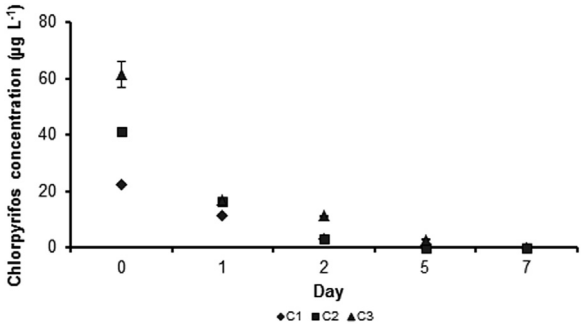

Fig. 1. Chlorpyrifos concentrations (Mean ± SD) in water in each treatment at initial time (day 0) and in the different sampling days. Initial concentrations (µg chlorpyrifos L^{-1}): C1 (rhombus): 22.4 ± 0.15; C2 (square): 41.25 ± 1.91; C3 (triangle): 61.4 ± 4.71.

2.3. Sampling and chromatographic analysis

Water samples were taken before chlorpyrifos application (to rule out the baseline presence of pesticides), just after chlopyrifos application (initial concentration), and 1, 2, 5, and 7 days after chlorpyrifos addition. Chlorpyrifos concentrations were measured by gas chromatography fitted with a standard electron capture and flame photometric detectors, GC-ECD (GC VARIAN 3400) according to Goncalvez and Alpendurada (2002), with minor modifications. Pestanal® chlorpyrifos were used. All of the solvents used were of pesticide-grade quality (Merck, Darmstadt, Germany). Pesticide concentrations were measured by duplicate. After 7 day, pesticide concentrations were below the detection limit of 0.1 µg chlorpyrifos L^{-1}. Crabs were sampled before the chlorpyrifos application (Day 0) and 8, 15, and 22 days after the chlorpyrifos application (when pesticide concentrations were below the detection limit). The sampled crabs (three for each replicate, a total of nine per group per day) were cryoanesthetized, measured and weighed. Samples of gills, hepatopancreas and ovary were dissected and the gonads were immediately weighed.

2.4. Histological analysis

The oocytes were photographed immediately after the samples were extracted (in fresh) under a stereoscopic microscope, with a millimeter scale used as reference. The mayor and minor axes were measured using the TPS dig program. Oocyte volume was calculated using the formula $Vo = 1/6\ (a \cdot b^2 \cdot \pi)$, where a = major axis and b = minor axis (Corey and Reid, 1991). The proportion of atretic oocytes was calculated as the ratio of atretic oocyte type /100 oocytes counted. The atretic oocyte index was calculated in each individual, counting the proportion of atretic oocytes in a total of 25 oocytes randomly chosen from four different ovarian regions (a total of 600 oocytes observed per

group per day). The proportion of atretic oocytes was obtained using the formula % atretic oocytes =(atretic oocyte number/total oocyte number)×100. Gonadosomatic indexes (GSI) were calculated according to the formula: GSI=(ovarian wet weight/body wet weight)×100 (Medesani et al., 2004).

The gills related to the third pair of pereiopods were dissected, whereas the gills related to the first two pairs of pereiopods were not used due to the flatworm (*Temnocephala* sp.) eggs attached to them (Dioni, 1967). Hepatopancreas samples were taken from each side of the crab, fixing in Bouin solution for 4 h and preserved in a 70% ethanol solution. Tissues samples were dehydrated in ethanol series, washed in xylene, and embedded in Histoplast®. Tissue sections were cut in 6 μm sections and stained with hematoxylin-eosin. The samples were observed under a light microscope.

In gills, pillar cell disruption (collapsed lamellae) and hyperplasia were analyzed. The lamellae of the gills were considered as affected if any of these histopathologies were observed. The proportions of hyperplasias, collapsed lamellae and affected lamellae/total lamellae per gill were calculated. In the hepatopancreas, several tubules (from 5 to 10) were present in every section. Four tubules were observed by slide (36 total tubules for every day and treatment). The observed tubules were randomly selected by using a grid. Once the point was selected, the entire tubule was observed. The F and B -cell types were identified and counted. Histopathologies as abnormal lumen and necrosis were counted (Al-Mohanna and Nott, 1989; Icely and Nott, 1992; Bhavan and Geraldine, 2000). The proportion of affected tubules (tubules where histopathologies were observed) was calculated and compared between exposed and control crabs.

2.5. Statistical analysis

The proportion of hyperplasia, collapsed lamellae and affected lamellae per gill, the number of F and B -cells per tubule, the GSI, the oocyte volume and the proportion of atretic oocytes of exposed and control crabs were compared using the Kruskal–Wallis test followed by Dunn's post-hoc test, since the data were not normally distributed or heteroscedastic. The number of affected tubules in the hepatopancreas was compared using the chi-square goodness of fit test, where the control group data were used as expected value and compared with the values observed at the different concentrations. In all statistical tests, a significance level of 5% was adopted (Zar, 1996).

3. Results

3.1. Experimental conditions

Chlorpyrifos concentrations in water were higher after the application, but their concentrations were quickly reduced, and after seven days they were too low to be quantified (Fig. 1). The values of temperature, dissolved oxygen, pH and conductivity were 16 ± 3.69 °C, 7.06 ± 0.89 mg l^{-1}, 7.08 ± 1.56 and 1311.86 ± 9.83 μS/cm, respectively. The temperature varied during the day, but there were no statistically significant differences between the groups. No mortality was observed throughout the experiments.

3.2. Effects of chlorpyrifos on the gills

The histopathological lesion most frequently observed was the disruption of pillar cells, which resulted in the collapse of the lamellae. Hyperplasia was also observed, resulting in the formation of a globular lamellae and the reduction in the interlamellar space. These histopathologies were observed 8, 15 and 22 days after the pesticide input, even if concentrations in water were not detected (Fig. 2).

The hyperplasia proportion in crabs exposed to C1 increased on all the days evaluated, whereas that in those exposed to C2 increased on day 15 and that in those exposed to C3 increased on day 8. The number of collapsed lamellae and affected lamellae increased on all the day evaluated and at all the concentrations ($p < 0.05$), except in crabs exposed to C2 on day 15 (Fig. 3).

3.3. Effects of chlorpyrifos on the hepatopancreas

3.3.1. F -cells

The number of F -cells in the control group did not vary; there were no differences in the number of F -cells on any day.

On day 8, there were no differences in the number of F -cells between the crabs from the control group and those exposed to chlorpyrifos. On day 15, there was a decrease in the number of F -cells of crabs exposed to C1 and an increase in those crabs exposed to C3. On day 22, there was an increase in the number of F -cells at the three concentrations evaluated ($p < 0.05$) (Figs. 2 and 4).

3.3.2. B -cells

In the control group, the number of B -cells decreased after 15 and 22 days ($p < 0.05$). On day 8, there were no differences in the number of B -cells between the crabs exposed to chlorpyrifos and those from the control group. There was a significant increase in the number of B -cells in crabs exposed to C3 on day 15 and a significant increase in the number of B -cells at the three concentrations evaluated on day 22 ($p < 0.05$) (Figs. 2 and 4).

The histopathology most frequently observed was abnormal lumen, followed by necrosis and delamination of the epithelium (Rodríguez et al., 1994; Bhavan and Geraldine, 2000; Pinho et al., 2003; Sousa et al., 2005; Bianchini and Monserrat, 2007) (Fig. 2). The number of affected tubules was not different between control and exposed crabs on day 8. However, there was an increase in the number of affected tubules in crabs exposed to C3 on day 15 and an increase in the proportion of affected tubules on day 22 at the three concentrations tested (Fig. 2 and 4).

3.4. Effects of chlorpyrifos on the gonads

3.4.1. Gonadosomatic indexes

All the females were in the exogenous vitellogenesis stage, characterized by orange oocytes. In the control group, the gonadosomatic (ovarian) indexes increased from 4.6 to 6.2 (± 0.9) (± 1.2). In exposed crabs, the gonadosomatic indexes varied between day 8 and day 22. However, there were no significant differences between the control and the exposed crabs on any day (Fig. 5).

3.4.2. Oocyte volume

In the control group, the oocyte volume increased from 2.92 (± 0.74) mm^3 on day 8 to 3.46 (± 0.92) mm^3 on day 22. In general, there were almost no effects of the exposure to chlorpyrifos on the oocyte volume. There was an increase in oocyte volume of crabs exposed to C1 only on day 8 ($p < 0.05$), but there were no differences in days 15 and 22 (Fig. 5).

3.4.3. Proportion of atretic oocyte

On day 8 there was an increase in the proportion of atretic oocytes (white colored, smaller and irregular oocytes) in crabs exposed to every concentration. However, there were no differences on day 15, but there was an increase in the proportion of atretic oocytes in crabs exposed to C2 and C3 on day 22 ($p < 0.05$) (Figs. 2 and 5).

4. Discussion

The chlorpyrifos concentrations used at the beginning of the experiment may be higher than those found in the environment (Schulz, 2001; Jergentz et al., 2005; Marino and Ronco, 2005). However, these concentrations quickly decreased until they were not found (Quantification limit: 0.1 μg L^{-1}), as it might occur in the

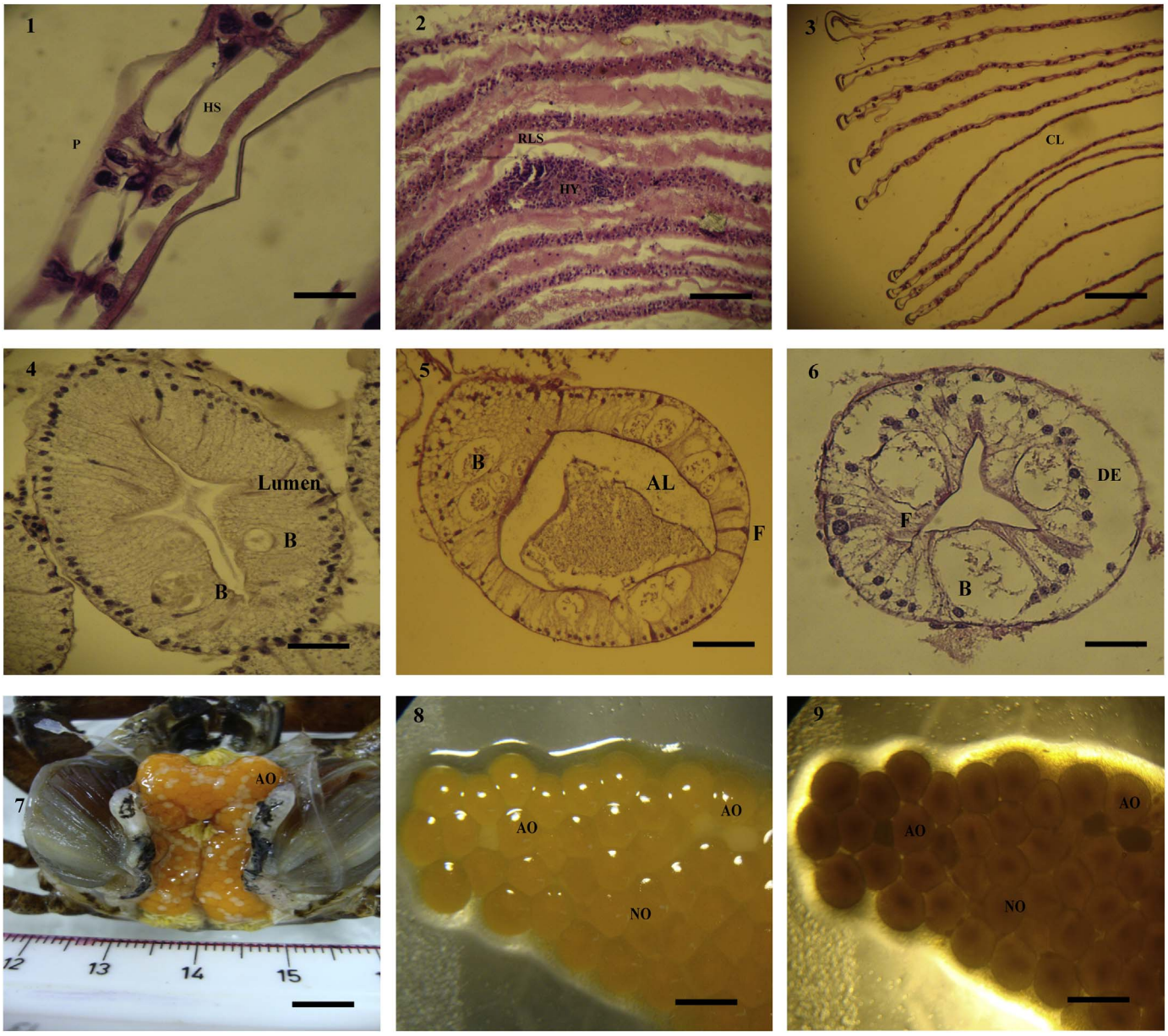

Fig. 2. Histological features of the gills, hepatopancreas and gonads of *Zilchiopsis collastinensis* female crabs exposed to different concentrations of chlorpiryfos. (1) Normal lamellae of Zilchiopsis collastinensis of the control group, showing regular size, unaffected pillar cells (P) and normal hemocoelic space (HS). Bar 0.01 mm. (2) Affected lamella from crabs initially exposed to C_1 after 8 days of exposure showing hyperplasia (HY), which reduced the inter-lamellar space (RLS). Bar 0.05 mm. (3) Collapsed lamellae (CL) after disruption of pillar cells from crabs initially exposed to C_1 after 15 days of exposure. Bar 0.1 mm. (4) Tubule of a control crab, showing normal lumen with star appearance. Bar = 0.03 mm. (5) Tubule of crab exposed to C_3 after 15 days, with abnormal lumen (AL) and proliferation of deep stained F cells and large vacuolated B cells. Bar = 0.03 mm. (6) Abnormal lumen of a crab exposed to C3, where delamination of the epithelia (DE) is observed. Bar = 0.03 mm. (7) Macroscopic observation of mature gonads of *Zilchiopsis collastinensis*, characterized by orange oocytes and some white atretic oocytes (AO). Observe the inverted U shape gonad, proper of crabs from the trichodactylidae family. Bar = 0.5 cm. (8) and (9) Ovary piece observed with reflected and transmitted light respectively, where atretic oocytes (AO) are differentiated from the normal oocytes (NO). Bar = 2 mm.

environment if the sampling is carried out several days after pesticide inputs (after spraying and/or rain event). Besides no lethal effects were observed, there were some effects on the hepatopancreas, gonads and gills at tissue scale, which were observed even after pesticide concentrations were not detected in water.

Gills are the first organ in contact with biocides present in the water and are often damaged or modified by exposure to toxicants. Histological effects should be observed first in this organ, before the pollutants reach the internal organs (Newman and Unger, 2003). In the present study, the gills were quickly affected, at least faster than the hepatopancreas, as histopathological effects were observed ot day 8 and continued to be observed in time.

In previous study carried out in *Zilchiopsis collastinensis* crabs exposed to endosulfan, gills appeared to recover themselves after the pesticide concentrations were no longer detected in the water (Negro, 2015). Although in the present study we expected the same behavior when crabs were exposed to chlorpyrifos, the histopathologcial effects continued to be observed for several days after the end of exposure. So, deleterious effects, especially those related to gas exchange, may remain several days after chlorpyrifos concentrations decrease.

The crab hepatopancreas is a dynamic organ related to food digestion and storage of energy reserves. However, its role is not restricted only to digestive functions.

The hepatopancreas is the main organ of detoxification, where different systems like cytochrome P450, lysosomes and metallothionein-like proteins are detected (Vogt, 2002). One of the mechanisms

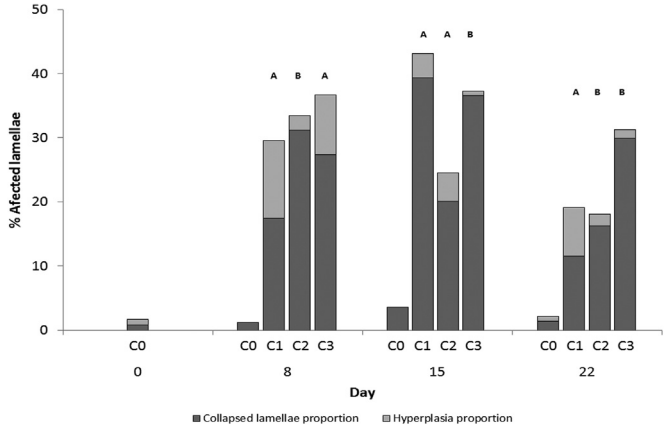

Fig. 3. Percentage proportion of collapsed lamellae (dark grey), hyperplasia (light grey) and affected lamellae (entire column) of crabs exposed to different chlorpyrifos concentrations at different days. A: Significant differences in the proportion of hyperplasias, collapsed lamellae and affected lamellae. B: Significant differences in the proportion of collapsed lamellae and affected lamellae ($p < 0.05$).

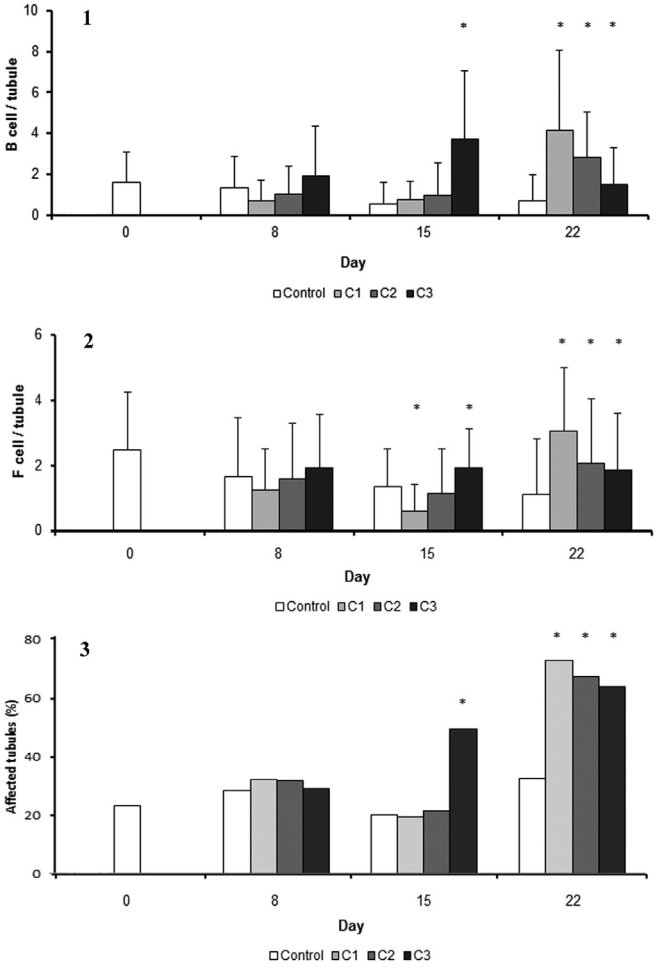

Fig. 4. (1) Mean (+SD) cell number of F cell per hepatopancreatic tubule (cross section) of *Zilchiopsis collastinensis* crabs exposed to different chlorpyrifos concentrations at different days. (2) Mean (+SD) cell number of B cell per hepatopancreatic tubule (cross section) of *Zilchiopsis collastinensis* crabs exposed to different chlorpyrifos concentrations at different days. (3) Percentage proportion of affected hepatopancreatic tubules of *Zilchiopsis collastinensis* crabs exposed to different chlorpyrifos concentrations at different days. N = 30 tubules observed per concentration and day. (*) = significantly different from the control group ($p < 0.05$).

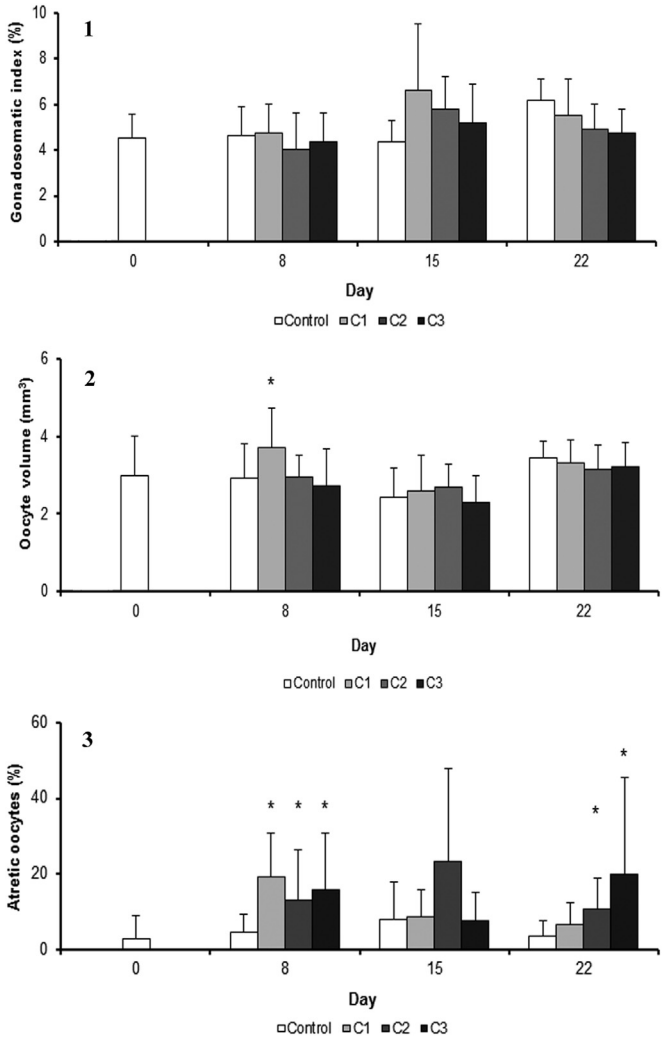

Fig. 5. (1) Gonadosomatic indexes of *Zilchiopsis collastinensis* crabs exposed to different chlorpyrifos concentrations at different days. (2) Oocyte volume of *Zilchiopsis collastinensis* crabs exposed to different chlorpyrifos concentrations at different days. (3) Percentage of atretic oocytes of *Zilchiopsis collastinensis* crabs exposed to chlorpyrifos at different days. (*) = significantly different from the control group ($p < 0.05$).

involved in this detoxification is the F and B cell complex. The hepatopancreas is composed of E, F, B and R -cells. The E -cells are precursor cells and are characterized by a nucleus that occupies most of the cell volume. They are unspecialized and cell division is confined to this type of cell. R or F -cells are produced according to different stimuli. R -cells are characterized by numerous small vacuoles which are full of lipids and reflect the nutritional status (Al-Mohanna and Nott, 1989; Icely and Nott, 1992). F -cells are the sites of enzyme synthesis and are characterized by the proliferation of the endoplasmic reticulum (Vogt, 2002). According to the scheme proposed by Hirsch and Jacob, these cells then transform into B -cells (Icely and Nott, 1992). B -cells secrete holocrine, especially under pollutant stress. It has been considered that these cells reflect the remnants of intracellular digestion and a later stage of an F -cell which produces the digestive enzymes. In aging B -cells, there are fusion events of the lysosomal compartment into one large vacuole, before it is eliminated from the epithelium and incorporated into feces. The transitional stage between F and B -cells (F/B transitional cells) are full of vesicles and lysosomes. Köhler et al. (1998) observed the expression of P-glycoprotein (*p*-gp) in the microvilli of transitional F/B cells in the hepatopancreas of *Carcinus maenas*, forming a first line of defense against the uptake of toxins. The fact that P-gp expression is specifically restricted to transitional F/B cells implies that these cells are specialized in accumulating and

eliminating toxic compounds.

In this case, the exposure of *Z. collastinensis* crabs to chlorpyrifos caused an increase in F and B -cells, which are related to detoxification and debris excretion. Sreeram and Menon (2005) found that the exposure of the shrimp *Metapenaeus dobsoni* to petroleum hydrocarbons also caused an increase in the number of F, F/B and especially B -cells, indicating a high rate of excretion from the hepatopancreas. In a previous study with *Z. collastinensis* exposed to endosulfan, imbalances in the F and B -cells were also observed, even after pesticide concentrations were no longer detected in the water (Negro, 2015). In the present study, the histopathological effects were observed after the effects occurred at a lower organization level, being the changes in detoxification related to cell number able to be observed even after the pesticide concentrations in water were not detected.

The number of affected tubules may be related to this high excretion rate. The main histopathology observed was the abnormal lumens. The increase in the proportion of abnormal lumens fits quite well with the number of F and B -cells per tubule, i.e., they increased on day 15 in crabs exposed to C3 and at all the concentrations after 22 days. The increase in F -cells might be related with the enzyme production, indicating that detoxification processes may be still in progress. The increase in the number of B -cells is also related to the excretion from the hepatopancreas (Sreeram and Menon, 2005), which is in turn related to open (secretor) lumens, indicating a high excretion rate of material to the feces.

Oocyte growth is closely related to the hepatopancreas during secondary vitellogenesis because this organ is the main source of egg yolk proteins (Harrison, 1990). The increase in detoxification processes as the F – B cells may decrease the energy available for oocyte growth. On the one hand, some energy is needed to maintain the detoxification processes and if these processes increase, the energy expenditure increases. On the other hand, the increase in the number of F and B -cells may be in detriment of the number of R -cells, as observed in *Z. collastinensis* crabs exposed to endosulfan (Negro, 2015). These cells take up material from the lumen by endocytosis and provide the main storage site for carbohydrates, lipids and glycogen (Icely and Nott, 1992). A decrease in their number may reduce the energy uptake from the food and storage, reducing the total energy amount and, eventually, the delivery of lipoproteins to the ovaries.

Exposure to metals and pesticides may reduce the oocyte size and thus the gonadosomatic index, as observed by different authors in the crabs *Chasmagnathus granulata*, *Uca pugilator*, *Somanniathelphusa pax* and *Geothelphusa dehaani* (Rodríguez et al., 1994, 2000; Medesani et al., 2004; Yamaguchi et al., 2008). According to these authors, this reduction might be related to a reduction in carbohydrates (a decrease in energy reserves) and possible effects on the gonad-inhibiting hormone. In the present study, we found no differences in ovarian indexes between exposed and control crabs and almost no effects on the volume of regular oocytes. Thus, the energy reserves might not be reduced and/or the gonad-inhibiting hormones might not be affected in our system, where exposure was not permanent. However, we observed an increase in the number of atretic oocytes, which were white, smaller than regular oocytes and occurred during exogenous vitellogenesis (Rodríguez et al., 1994). These affected oocytes are no longer available to be fertilized and resorption processes occur.

Besides the decrease in the number of available oocytes and eventually in the number of eggs and neonates, this “strategy” of reducing the number of available oocytes instead of reducing the growth of the entire gonad, also observed when this crab was exposed to endosulfan, might be an adaptation of this species to the environment. *Zilchiopsis collastinensis* is a crab whose reproductive cycle is closely related to the flood pulse of the middle Paraná River. The females develop their gonads from January to October, and spawning occurs at the beginning of the spring. They incubate their eggs in the abdomen from October to December, when hatching occurs. Hatching is synchronized with the flood peak in summer (December), when the river gets into the floodplains. The new availability of habitat and food increases the survival chance of the offspring in the juvenile stage. A general delay in oocyte growth, as observed in *Uca pugilator* and *Chasmagnathus granulata* exposed to metals and pesticides respectively (Rodríguez et al., 1994, 2000), might desynchronize the hatching with the flood, potentially increasing the predation of juveniles because of the reduction of habitats and/or trophic offer. Facing a stressing situation that depletes energy reserves, caused by both reduction in energy uptake in hepatopancreas and an increased energy demand because of defence mechanisms, these crabs may reabsorb the lipids from some oocytes without altering the development of others, as was observed in a previous work with this species (Negro, 2015).

Considering the effects caused by the exposure to chlorpyrifos in a biomarker vision, the number of collapsed lamellae in gills, the modification in the F-B complex and the abnormalities in the lumen in the hepatopancreas, and the number of atretic oocytes in gonads might be used to indicate contamination from agricultural zones and affected sites, especially related with exposure to pesticides as chlorpyrifos (this work) or endosulfan (Negro, 2015). As these hispathological effects are persistent in time, they might be used to recognize affected sites even after the pesticide concentrations are not easy to be detected by chemical methods.

Acknowledgments

This study was supported by the grants CONICET Argentina 2012–2014, PIP No 11220110100998CO Crustáceos decápodos del sistema del Plata como indicadores de estrés ambiental. Director: Pablo Collins.

References

Abad-Rosales, S.M., Frías-Espericueta, M.G., Inzunza-Rojas, A., Osuna-López, I., Páez-Osuna, F., Lozano-Olvera, R., Voltolina, D., 2010. Histological effects of Cu^{2+} to white shrimp *Litopenaeus vannamei* (Crustacea: decapoda) juveniles at low salinities. Rev. Biol. Mar. Y. Ocean 45 (1), 99–105.

Al-Mohanna, S.Y., Nott, J.A., 1989. Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: decapoda) during the moult cycle. Mar. Biol. 101, 535–544.

Bhavan, P.S., Geraldine, P., 2000. Histopathology of the hepatopancreas and gills of the prawn *Macrobrachium malcolnsonii* exposed to endosulfan. Aquat. Toxicol. 50, 331–339.

Bhavan, P.S., Geraldine, P., 2009. Manifestation of carbaryl toxicity on soluble protein and histopathology in the hepatopancreas and gills of the prawn, *Macrobrachium malcolmsonii*. J. Environ. Biol. 30 (4), 533–538.

Bianchini, A., Monserrat, J.M., 2007. Effects of methyl parathion on *Chasmagnathus granulatus* hepatopancreas: protective role of Sesamol. Ecotoxicol. Environ. Saf. 67, 100–108.

Collins, P., Williner, V., Giri, F., 2007. Littoral communities. In: Iriondo, M.H., Paggi, J.C., Parma, J. (Eds.), The Middle Paraná River. Limnology of a subtropical Wetland. Springer-Verlag, Heidelberg, pp. 277–301.

Corey, S., Reid, D.M., 1991. Comparative fecundity of decapod crustaceans, I. The fecundity of thirty tree species of nine families of caridean shrimps. Crustaceana 60 (3), 270–294.

Dioni, W., 1967. Temnocephalas argentinas. Acta Zool. Lillo. 23, 349–360.

Ernst, W.R., Jonah, P., Doe, K., Julien, G., Hennigar, P., 1990. Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. Environ. Toxicol. Chem. 10, 103–114.

Goncalvez, C., Alpendurada, M.F., 2002. Multiresidue method for the simultaneous determination of four groups of pesticides in ground and drinking waters, using solid-phase microextraction-gas chromatography with electron-capture and thermionic specific detection. J. Chromatogr. A 968, 177–190.

Harrison, K.E., 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. J. Shell Res. 9 (1), 1–28.

Hinton, D.E., Laurén, D.J., 1990. Integrative histopathological approaches to detecting effects of environmental stressors on fishes. Am. Fish. Soc. Symp. 8, 51–66.

Icely, J.D., Nott, J.A., 1992. Digestion and absorption: digestive system and associated organs. In: Harrison, F.W., Humes, A.G. (Eds.), Microscopic Anatomy of Invertebrates, Vol. 10: Decapod Crustacean. Wiley-Liss Inc, New York, pp. 147–201.

Jergentz, S., Mugni, H., Bonetto, C., Schulz, R., 2005. Assesment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. Chemosphere 61, 817–826.

Köhler, A., Lauritzen, B., Jansen, D., Böttcher, P., Teguliwa, L., Krüner, G., Broeg, K., 1998. Detection of P-glycoprotein mediated MDR/MXR in *Carcinus maenas* hepatopancreas by immuno-gold-silver labeling. Mar. Environ. Res. 46 (1–5), 411–414.

Lubzens, E., Khayat, M., Ravid, T., Funkenstein, B., Tietz, A., 1995. Lipoproteins and lipid accumulation within the ovaries of penaeid shrimp. Isr. J. Aquacult.-Bamidgeh 47 (3–

4), 185–195.
Marino, D., Ronco, A., 2005. Cypermethrin and Chlopyrifos concentration levels in surface water bodies of the Pampa Ondulada, Argentina. Bull. Environ. Contam. Toxicol. 75, 820–826.
Medesani, D.A., López Greco, L.S., Rodríguez, E.M., 2004. Interference of cadmium and copper with the endocrine control of ovarian growth, in the estuarine crab *Chasmagnathus granulata*. Aquat. Toxicol. 69, 165–174.
Mortimer, M.R., 2000. Pesticide and trace metal concentrations in Queensland estuarine crabs. Mar. Poll. Bull. 41 (7–12), 359–366.
Mugni, H., Paracampo, A., Demetrio, P., Pardi, M., Bulus, G., Ronco, A., Bonetto, C., 2016. Toxicity persistence of Chlorpyrifos in runoff from experimental soybean plots to the non-target amphipod *Hyalella curvispina*: effect of crop management. Bull. Environ. Contam. Toxicol. 70 (2), 257–264.
Negro, C.L., Senkman, L.E., Marino, F., Lorenzatti, E., Collins, P., 2014. Effects of chlorpyrifos and endosulfan on different life stages of the freshwater burrowing crab *Zilchiopsis collastinensis* P.: protective role of chorion. Bull. Environ. Contam. Toxicol. 92, 625–630.
Negro, C.L., 2015. Histopathological effects of endosulfan to hepatopancreas, gills and ovary of the freshwater crab *Zilchiopsis collastinensis* (Decapoda: trichodactylidae). Ecotoxicol. Environ. Saf. 113, 87–94.
Newman, M.C., Unger, M.A., 2003. Fundamentals of Ecotoxicology. Lewis publishers, CRC Press, Florida.
Pandey, S., Parvez, S., Ansari, R.A., Ali, M., Kaur, M., Hayat, F., Ahmad, F., Raisuddin, S., 2008. Effects of exposure to multiple trace metals on biochemical, histological and ultraestructural features of gills of a freshwater fish, *Channa punctata* Bloch. Chem. Biol. Interact. 174, 183–192.
Pinho, G.L.L., da Rosa Moura, C., Yunes, J.S., Luquet, C.M., Bianchini, A., Monserrat, J.M., 2003. Toxic effects of microcystins in the hepatopancreas of the estuarine crab *Chasmagnathus granulatus* (Decapoda, Grapsidae). Comp. Biochem. Physiol. C 135, 459–468.
Reddy, P.S., Bhagyalakshmi, A., Ramamurthi, R., 1983. Effects of sumithion on ovarian growth of a fresh water rice field crab (*Oziotelphusa senex senex* Fabricius). Toxicol. Let. 18, 273–276.
Reish, D.L., Oshida, P.S., 1987. Short-term bioassay. In: FAO Fish Technical Paper Part 6, Manual of Methods in Aquatic Environment Research. 247, pp. 1–62.
Rodríguez, E.M., Schuldt, M., Romano, L., 1994. Chronic histopatological effects of paratión and 2,4-D on female gonads of *Chasmagnathus granulata* (Decapoda, Brachyura). Food Chem. Toxicol. 32, 811–818.
Rodríguez, E.M., López Greco, L.S., Fingerman, M., 2000. Inhibition of ovarian growth by cadmiun, in the fiddler crab *Uca pugilator* (Decapoda, Ocypodidae). Ecotox. Environ. Saf. 46, 202–206.
Schulz, R., 2001. Rainfall-induced sediment and pesticide input from orchards into the Lourens river, Western Cape, South Africa: Importance of a single event. Wat. Res. 35 (8), 1869–1876.
Senkman, L.E., Negro, C.L., Lopretto, E.C., Collins, P.A., 2015. Reproductive behaviour of three species of freshwater crabs of the family Trichodactylidae (Crustacea: decapoda) including forced copulation by males. Mar. Freshw. Behav. Physiol. 48 (2), 77–88.
Sousa, L.G., Cuartas, E.I., Petriella, A.M., 2005. Fine structural analysis of the epithelial cells in the hepatopancreas of *Palaemonetes argentinus* (Crustacea, Decapoda, Caridea) in intermoult. Biocell 29 (1), 25–31.
Sreeram, M.P., Menon, N.R., 2005. Histopathological changes in the hepatopancreas of the penaeid shrimp *Metapenaeus dobsoni* exposed to petroleum hydrocarbons. J. Mar. Biol. Assoc. India 47 (2), 160–168.
Tomlin, C.D.S., 1998. The Pesticide Manual, 11th ed. British Crop Protection Council, BCPC Publications Sales, UK.
Varó, I., Serrano, R., Pitarch, E., Amat, F., López, F.J., Navarro, J.C., 2000. Toxicity and bioconcentration of chlorpyrifos in aquatic organisms: *Artemia parthenogenetica* (Crustacea), *Gambusia affinis*, and *Aphanius iberus* (Pisces). Bull. Environ. Contam. Toxicol. 65, 623–630.
Vogt, G., 1987. Monitoring environmental pollutants such as pesticides in prawn aquaculture by histological diagnosis. Aquaculture 67, 157–164.
Vogt, G., 2002. Functional anatomy. In: Holdich, D.M. (Ed.), Biology of freshwater crayfish. Blackwell, Oxford, pp. 53–151.
Wu, J.P., Chen, H.C., Huang, D.J., 2008. Histopathological and biochemical evidence of hepatopancreatic toxicity caused by cadmium and zinc in the white shrimp, *Litopenaeus vannamei*. Chemosphere 73, 1019–1026.
Yamaguchi, S., Celino, F.T., Ito, A., Agusa, T., Tanabe, S., Tuyen, B.C., Miura, C., Miura, T., 2008. Effects of arsenic on gonadal development in freshwater crab, *Somanniathelphusa pax*, in Vietnam and *Geothelphusa dehaani* in Japan. Ecotoxicology 17, 772–778.
Zar, J.H., 1996. Biostatistical Analysis. Prentice Hall, New York.