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Effects of Glyphosate on Somatic and Ovarian Growth in the Estuarine Crab *Neohelice granulata*, During the Pre-Reproductive Period

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Abstract Adult females of the estuarine crab *Neohelice granulata* were exposed during the 3-month pre-reproductive period (winter) to the herbicide glyphosate, the most used pesticide in Argentina, at three different concentrations (0.02, 0.2, and 1 mg/L, as active ingredient). At the end of the in vivo assay, the body weight gain and the ovarian growth were estimated, the last one in terms of the gonadosomatic index (GSI), the relative proportion of the different kind of oocytes, and their relative size. A decrease in the body weight gain was observed by effect of pure glyphosate, at all concentrations assayed. Although no differences in either the GSI or vitellogenic protein content of the ovary were noted between any glyphosate concentration and control, a higher proportion of reabsorbed vitellogenic oocytes was observed in the ovaries of crabs exposed to glyphosate at 1 mg/L, together with an increased area of previtellogenic oocytes. These effects were confirmed in vitro, at a glyphosate concentration of 0.2 mg/L. In fact, a higher area of previtellogenic oocytes was seen when glyphosate was added to the culture medium containing ovarian tissue, but a significant higher incidence of reabsorbed vitellogenic oocytes was seen only when eyestalk tissue was also added to the vials, suggesting that the secretion of some neurohormone involved in reabsorption is enhanced. The obtained results indicate that glyphosate is able to harm, in the studied species, both somatic and the ovarian growth.

Keywords Estuarine crabs · Glyphosate · Reproduction · Ovarian growth · Oocytes

1 Introduction

Glyphosate [N-(phosphonomethyl) glycine] is currently the most widely used herbicide worldwide (Mesnage et al. 2015). In Argentina, glyphosate is, by far, the herbicide intensively applied on several crops (Arancibia 2013; Brodeur et al. 2011). This is a nonselective systemic herbicide that reduces plant growth by acting as an inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is involved in the synthesis pathway of aromatic amino acids present in plants and microorganisms, but not in animals (Carlisle and Trevor 1988; Lydon and Duke 1989). In Argentina, several commercial formulations of glyphosate are currently applied on crops; currently, more than 20 million hectares of soybean are treated with this herbicide (Leguizamón 2014). As a consequence, environmental levels of glyphosate have been reported to range between 0.1 and 0.7 mg/L in water and between 0.5 and 5 mg/kg in sediment (Peruzzo et al. 2008).

Crustaceans represent a group of invertebrates widely distributed in marine, estuarial, and freshwater environments. The crab *Neohelice* (=*Chasmagnathus*) *granulata* (Decapoda, Brachyura) lives in several

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estuarine environments along the Atlantic coast of both Argentina and Brazil. In Argentina, this species inhabits the entire coast of Samborombón Bay, corresponding to the external zone of the "Rio de la Plata" estuary, which is strongly influenced by tides. Both adult and juvenile *N. granulata* crabs live in the meso- and supralittoral zones of the coast, forming very dense populations. The reproductive cycle of *N. granulata* comprises three periods (López Greco and Rodríguez 1999): pre-reproductive (winter), during which the ovary grows and mature; reproductive (spring and summer), when both spawning and hatching occur; and finally the post-reproductive period (autumn), which leads to both ovarian quiescent and molting of adults. During the pre-reproductive period, the ovary enters into secondary vitellogenesis, uptaking the vitellogenin synthesized in the hepatopancreas and secreted to the hemolymph (Charmantier et al. 1997; Nagaraju 2011). After spawning, hatching, and larval development, the megalopa returns to the coast, molting successively to several juvenile instars and finally reaching the adult condition (López Greco and Rodríguez 1999).

A heavy charge of herbicides and other pesticides is carried by several rivers and channels that cross extensive agricultural areas and finally reach the Samborombón Bay (Comisión Administrativa del Río de La Plata 1990). *N. granulata* has been previously used as a model for ecotoxicological studies concerning the effects of several pesticides and heavy metals on embryonic development and larvae hatching (Sánchez et al. 2005; Zapata et al. 2001; Rodríguez and Pisanó 1993). As in other crustacean species, though, few studies about the disrupting effects of pesticides on the hormonal control of reproduction have been published (Rodríguez et al. 2007; LeBlanc 2007; Hyne 2011, for reviews). However, significant deleterious effects of glyphosate on the synthesis of several sexual steroids have been also reported in different animal models (Quasinti et al. 2009; Richard et al. 2005; Mesnage et al. 2015).

The present study was aimed at evaluating the ovarian growth, as well as some metabolic parameters related to the ovarian maturation, in *N. granulata* adult females exposed to 0.02, 0.2, and 1 mg/L of glyphosate during the pre-reproductive period. To this purpose, long-term in vivo assays were carried out with pure glyphosate. In order to investigate some effects, in vitro experiments were also carried out.

2 Materials and Methods

2.1 In Vivo Experiment

Adult females of *N. granulata* were randomly collected in June (body weight = 10.35 ± 0.12 g, $N = 75$), at the southern edge of Samborombón Bay, a non-polluted area at the mouth of the Río de la Plata estuary, Argentina. All bioassays were conducted in semi-static conditions according to the standard procedures recommended by the American Public Health Association (2005). Briefly, water from all test recipients was completely renewed twice a week, always maintaining the water quality parameters and laboratory conditions mentioned below.

Concentrated stock solutions of pure glyphosate (as acid, 99.8% purity; Sigma, Missouri) were prepared weekly by dissolving the appropriate amount of the chemicals in distilled water, to avoid precipitation. Small aliquots from these stock solutions were then added to the test recipients, previously filled with the dilution saline water detailed below. Nominal concentrations of 0.02, 0.2, and 1 mg/L were assayed; these concentrations had been shown to be sublethal (Avigliano et al. 2014a,b).

In order to validate nominal concentrations, water samples (15 mL) were taken at 0 and 72 h, i.e., the period for water replacement in all test containers. After derivatization at pH = 9 with 9-fluorenylmethylchloroformate (FMOC-CL), glyphosate concentrations were measured by high pressure liquid chromatography, coupled to a mass spectrophotometry detector Agilent, model VL. A X-SELECT C_{18} chromatographic column was used. A mixture of MeOH/NH_4 5 mmM/9 mM was chosen as mobile phase, with a flow rate of 0.5 mL/min.

Fifteen females were assigned to each treatment, i.e., control (with no toxic added) or treated with glyphosate at 0.02, 0.2, or 1 mg/L. For all experiments, each female was isolated in a glass container filled with 400 mL of saline water prepared by diluting artificial seawater salts (Tetra Marine Salt Pro, US) in dechlorinated tap water (hardness: 80 mg/L as equivalents of CaCO3; final salinity, 12 g/L, pH = 8.0 ± 0.5) provided with constant aeration. The solution of each container was completely replaced twice a week. A temperature of 23 ± 1 °C and a photoperiod of 14:10 (L/D) were maintained in the aquarium. All animals were fed twice a week with pellets prepared in the laboratory (according to

Chaulet et al. 2012) in an amount equivalent to 5% of body mass, supplemented with *Elodea* sp. fresh leaves ad libitum.

Females remained exposed to all glyphosate concentrations or control for 90 days, i.e., throughout the entire pre-reproductive period (June 24 to September 23). In order to evaluate the ovarian growth prior to spawning, ovigerous females were not considered for further analysis. Females that molted during the experiment were also excluded, taking into account that the energy invested for molting could represent a disadvantage for the ovarian growth. Therefore, at the end of the assays, non-ovigerous and non-molted females were weighed to determine body weight (BW), which were later compared to the initial body weight, in order to estimate weight gain (WG) as ((final BW-initial BW)/initial BW) × 100. Animals were finally sacrificed after anesthetizing them in ice water, and both ovaries and hepatopancreas were dissected and weighed in order to determine the gonadosomatic (GSI) and hepatosomatic (HSI) index as GSI or HSI = (GW or HW)/final BW) × 100, where GW and HW are the gonad and hepatopancreas wet weight, respectively. In addition, both the GSI and HSI were measured in an initial sample of crabs (10.78 ± 0.05 g body weight, $N = 15$).

Dissected ovaries were fixed in Bouin solution for 4 h at room temperature, dehydrated in alcohol series, and finally embedded in paraplast. Then, 5-μm sections were prepared and stained with hematoxylin and eosin. Previtellogenic, intermediate, and vitellogenic oocytes were characterized according to their size and degree of basophilia (Rodríguez et al. 1994). To assess the proportions of the different kind of oocytes, a grid of 100 points was used, counting the number of points included in each oocyte type, at a 40× magnification; at least three ovarian sections from each animal, taken from the thickest part of the ovary, were examined by this procedure. Considering the ellipsoidal shape of oocytes, both the major (M) and minor (m) diameter of every oocyte showing its nucleus were measured in the same histological sections above mentioned, in order to estimate the oocyte area. For this, a micrometric ocular lens, calibrated against a Leitz Wetzlar plate with 1/100 mm spacing, was used. As in previous studies (Rodríguez et al. 1994), the oocyte area was calculated as $(\pi/4) \times M \times m$.

An ELISA assay was employed to determine the total content of vitellogenic proteins (Vg: vitellogenin and vitellins). A primary antibody against native Vg was obtained by inoculating rabbits with purified Vg, according to previous studies (Dreon et al. 2003; García et al. 2008). Anti-IgG from rabbit, conjugated with peroxidase (BIOARS Lab.), was used as the secondary antibody. Purified Vg in a 1/500 dilution was used to prepare the standard curve (0–210 ng). Fifty microliters of either the standard or sample was placed, in triplicate, in a 96-well plate (Nunc-Immunoplate Polisorp). Samples were previously diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH = 9.6). Both primary and secondary antibodies were diluted (1/500) in PBS + 0.05% Tween + 6% powder milk. Absorbance was measured in all wells at 415 nm, by using an ELISA-plates reader (BIO-Rad Lab., Model 680); 2-20-Azino-di-3-ethylbenzthiazoline sulfonic acid was used as chromogen.

2.2 In Vitro Experiments

During June, stock female crabs were used for the in vitro experiments. Ovarian explants (approximately 1 × 0.5 cm each) were incubated for 24 h in 2-mL vials, inside culture chambers held at 27 °C, constant darkness and with CO_2 at 5%. Medium 199 was prepared using powdered medium with L-glutamine and Earle's salts (Sigma Chemical Co.), dissolved in crustacean saline (Cooke et al. 1977), and modified to compensate for the salts already present in this culture medium. As in previous studies (Sarojini et al. 1997; Rodríguez et al. 2000), 6 mg of penicillin-G per 100 mL of medium was added, and the pH was adjusted to 7.4 with 0.5 N NaOH.

Ten females were assigned to each experimental series, each female providing a similar piece of ovary to every treatment (blocking design). One series consisted of only ovary, either with the addition of pure glyphosate at 0.2 mg/L or with no glyphosate added (control); a second series involved the same treatments but every eyestalk of each sacrificed female was also added, to either control or glyphosate-treated vial. To this, eyestalks were cut off at their articulation with cephalothorax and below the ommatidia; after gently dissecting the cuticle, the soft tissue was washed and assigned to vials. At the end of the 24-h incubation period, all ovarian pieces were fixed and processed for histological analysis, following the same methodology described for the in vivo assays.

2.3 Statistical Analysis

A one-way ANOVA followed by LSD pairwise comparisons (Sokal and Rohlf 1981) was used for testing differences between experimental groups concerning both the proportion of each oocyte type and the ovarian vitellogenin content. Logarithmic or angular transformation of data was eventually used when homogeneity of variances was not confirmed in raw data. Proportion of survival, molted or ovigerous females were compared between experimental groups by means of the Fisher exact test (Sokal and Rohlf 1981).

3 Results

Measured glyphosate concentrations were close to the nominal ones; no significant degradation seems to occur at 72 h of aging, i.e., before replacing all test solutions (Table 1). Mortality was low in almost all treatments (Table 2), although it rose up to 33.33% at the lowest glyphosate concentration ($p < 0.05$, compared to control). At the highest concentration assayed, the percentages of both molting and ovigerous females reached 20 and 26.67%, respectively, which were not different ($p > 0.05$) from control values (Table 2). All the females that became ovigerous during the experiment lost the spawned eggs during the first days of incubation; these eggs were not fertilized, according to the verification made under stereoscopic lupe (50×), indicating that used females had no spermatophores stored in their spermathecae.

Compared to control, WG was significantly ($p < 0.05$) lower at all glyphosate concentrations tested (Fig. 1). Although no significant differences ($p > 0.05$) were observed in either the gonsadosomatic index or hepatosomatic index, a tendency toward a reduction of GSI was observed as glyphosate concentration increased (Fig. 1). No significant differences ($p > 0.05$) were noted in both the vitellogenic protein content of the ovary (overall mean: 1969.11 ± 202.15 μg/g of ovary) and its circulating levels (overall mean: 91.93 ± 7.93 μg/mL of hemolymph).

Concerning the proportion of oocyte types in the ovary observed at the end of the in vivo experiment, a significantly ($p < 0.05$) higher proportion of reabsorbed vitellogenic oocytes was observed at the highest glyphosate concentration, together with a lower proportion of normal vitellogenic oocytes (Fig. 2). On the other hand, the area of normal previtellogenic oocytes was significantly ($p < 0.05$) augmented by effect of pure glyphosate at 1 mg/L (Fig. 3). The results of the in vitro experiments confirmed that previtellogenic oocytes reached an area significantly ($p < 0.05$) bigger than that of control, when glyphosate was added to the culture medium at a concentration of 0.2 mg/L (Fig. 4b). When eyestalk tissue was added, though, no significant differences ($p > 0.05$) were noted in oocyte's area, but a significant ($p < 0.05$) percentage of reabsorbed vitellogenic oocytes was noted (Fig. 4a).

Representative sections of the ovaries analyzed both in vivo and in vitro can be seen in Fig. 5. The reabsorption of vitellogenic oocytes by effect of glyphosate, observed in vivo, implied the participation of follicular cells to phagocyte the rest of yolk, as shown by Fig. 5b. This figure also shows some previtellogenic oocytes from the in vivo exposure to 1 mg/L of glyphosate of bigger size compared to control (Fig. 5a). Figure 5c, d shows the same comparison, between 0.2 mg/L of glyphosate and control, for the in vitro assay.

Table 1 Nominal and measured concentrations of glyphosate, used in the in vivo assay

Nominal concentration (mg/L)	Measured concentration (mg/L)		
	0 h	72 h	Overall mean
0.02	0.0186 0.0225	0.0299 0.0269	0.0245 ± 0.0025
0.2	0.1786 0.1795	0.2108 0.2604	0.2073 ± 0.0192
1	0.8502 0.9009	1.6673 1.6449	1.2658 ± 0.2256

Table 2 Molting, spawning and mortality of females at the end of the in vivo assay

Glyphosate concentration (mg/L)	Ni	% mortality	% molting	% ovígerous	Nf
0 (control)	15	0.00	6.67	13.33	12
0.02	15	33.33 *	13.33	6.67	7
0.2	15	6.67	13.33	20.00	9
1	15	6.67	20.00	26.67	7

Ni initial number of females, *Nf* final number, considering only the non-molted, non-ovigerous surviving (NM-NO) females. Mortality percentage is always referred to NM-NO females

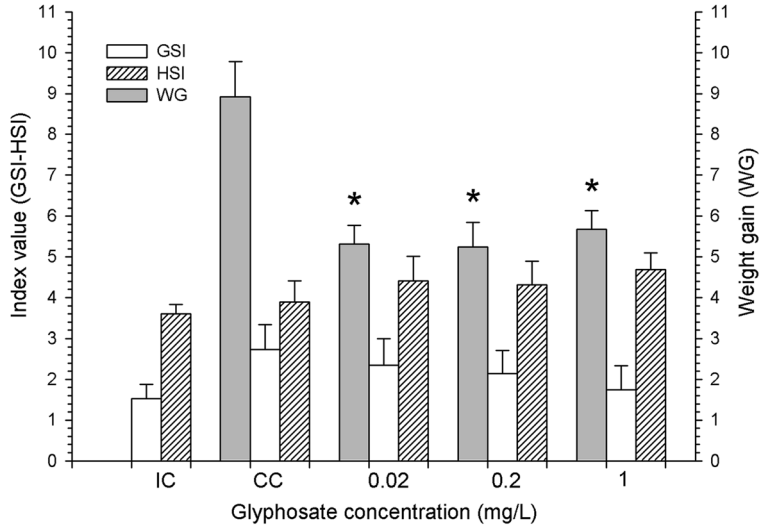

Fig. 1 Weight gain (WG), gonadosomatic (GSI), and hepatoxomatic (HIS) index of females at the end of the in vivo experiment. Number of females (Nf) is indicated in Table 2. IC = initial control; CC = concurrent control. Asterisks indicate significant differences ($p < 0.05$) with respect to control (CC)

4 Discussion

The accumulative effects of glyphosate on the ovarian growth of *N. granulata* could be observed at the end of the 3-month exposure period, which comprised the entire pre-reproductive period. This is a critical period for reproduction of the studied species, since the ovary is growing up to achieve the final maturity needed to spawn. The percentages of both molted and ovigerous females were mostly discrete and did not show differences between any treatment and control. Nevertheless, the exclusion of these animals for further analysis allowed us a more accurate estimation of the ovarian growth, which is certainly affected by both molting and recent spawning.

The reduction of somatic growth by effect of the three concentrations assayed of glyphosate was in accordance with previous results obtained in early juveniles of the crayfish *Cherax quadricarinatus*, who also showed a diminished body weight gain by effect of pure glyphosate, in correlation with decrease levels of both protein and lipid reserves (Avigliano et al. 2014a). Frontera et al. (2011) have reported similar results in advanced juveniles of *C. quadricarinatus*, by effect of

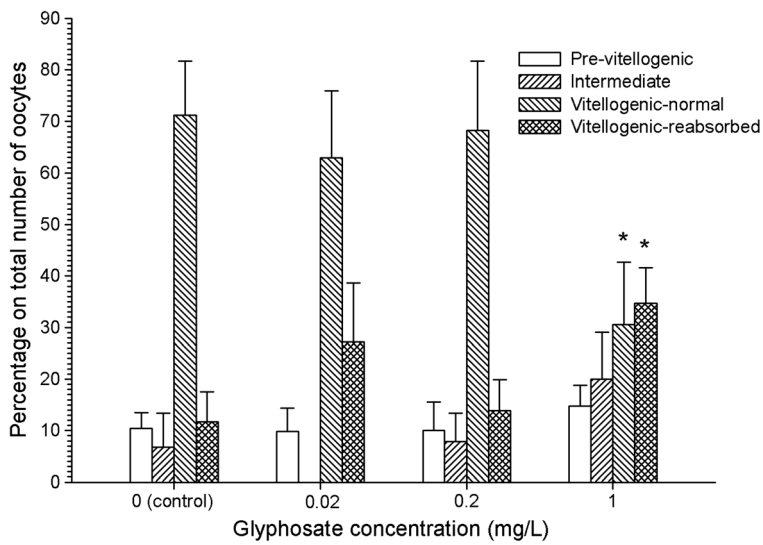

Fig. 2 Proportion of oocyte type in the ovary (mean ± SE) of females, at the end of the in vivo experiment. Number of females (Nf) is indicated in Table 2. Asterisks indicate significant differences ($p < 0.05$) with respect to control

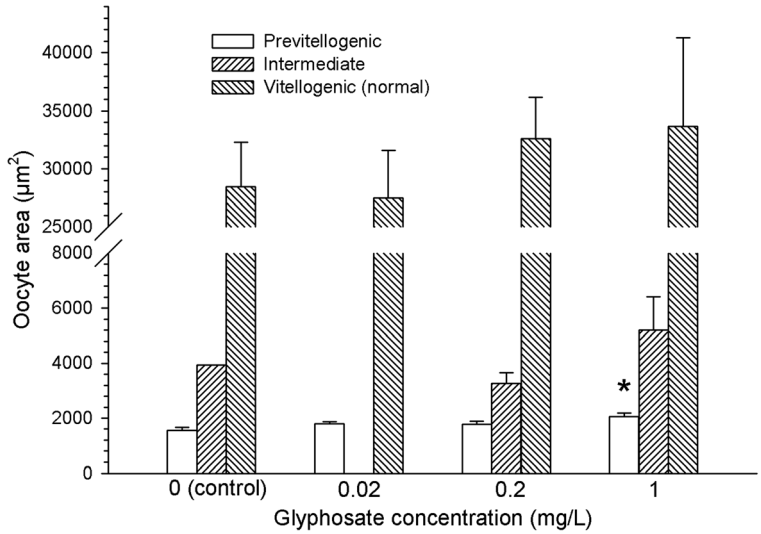

Fig. 3 Relative area of oocytes in the ovary (mean ± SE) of females, at the end of the in vivo experiment. Number of females (Nf) is indicated in Table 2. Asterisks indicate significant differences ($p < 0.05$) with respect to control

both pure glyphosate and a mixture of glyphosate with polyoxyethylenamine, the surfactant used in the original formulation of Roundup®. This glyphosate formulation also caused a somatic growth reduction in freshwater shrimps exposed for 40 days to concentrations ranging from 2.2 to 5.4 mg/L (Mensah et al. 2012). As suggested by these authors (Mensah et al. 2012; Frontera et al. 2011), the reduction of somatic growth could be related to the utilization of the energy needed to detoxify glyphosate.

Besides, it should be noted that a variety of undeclared surfactants have been used during the last years in several formulations of Roundup and other trademarks (Mesnage et al. 2015). In this sense, the results obtained in the current study are establishing a baseline of deleterious effects caused by pure glyphosate, which is valuable information for evaluating the toxicity of any commercial formulation of this herbicide.

Although neither the gonadosomatic index nor the ovarian Vg content were significantly affected by the compounds assayed, the histological analysis of the ovary showed a higher incidence of reabsorption in vitellogenic oocytes by effect of glyphosate at 1 mg/L. In a previous study made on *N. granulata* females, a reactional atresia leading to oocyte reabsorption was observed by effect of the herbicide 2,4-D, in combination with the commercial emulsionant nonylphenol (Rodríguez et al. 1994). A similar reactional atresia was in fact observed in the current study, according to the proliferation of the follicular cells inside the atretic oocyte.

In order to explain oocyte reabsorption observed in glyphosate-exposed crabs, a first possibility to be considered is that the yolk reabsorption could be a mechanism to compensate any reduction in the somatic growth of females, as reported for many other cases of stress (Sokolova et al. 2012). In fact, as mentioned before, a reduction of WG by effect of glyphosate took place at all the concentrations tested. However, oocyte reabsorption was only significant at the highest concentration tested. Therefore, a second possibility to be considered is that glyphosate could have caused some kind of endocrine disruption above some threshold concentration, which could have lead to ovarian reabsorption. Although no direct evidence is currently available to support this hypothesis, an increased reabsorption of vitellogenic oocytes was caused by glyphosate in the in vitro experiment, but only when eyestalk tissue was added. This result is suggesting that an imbalance in the secretion and/or transductional pathway of any neurohormone released by the eyestalks and related to the ovarian growth could be occurring by effect of glyphosate.

According to Charmantier (1997), growing of oocytes in the ovary is mainly determined by gradual decreasing in the gonad inhibiting hormone (GIH) secreted by the sinus gland at the eyestalks. If secretion of this inhibitory hormone increased when the ovary is undergoing the exogenous vitellogenesis (as in the current in vitro experiment), the reabsortion of vitellogenic oocytes could be explained. In addition, some other hormones related to GIH could be also participating in the ovarian reabsorption, both in vivo and in vitro. In

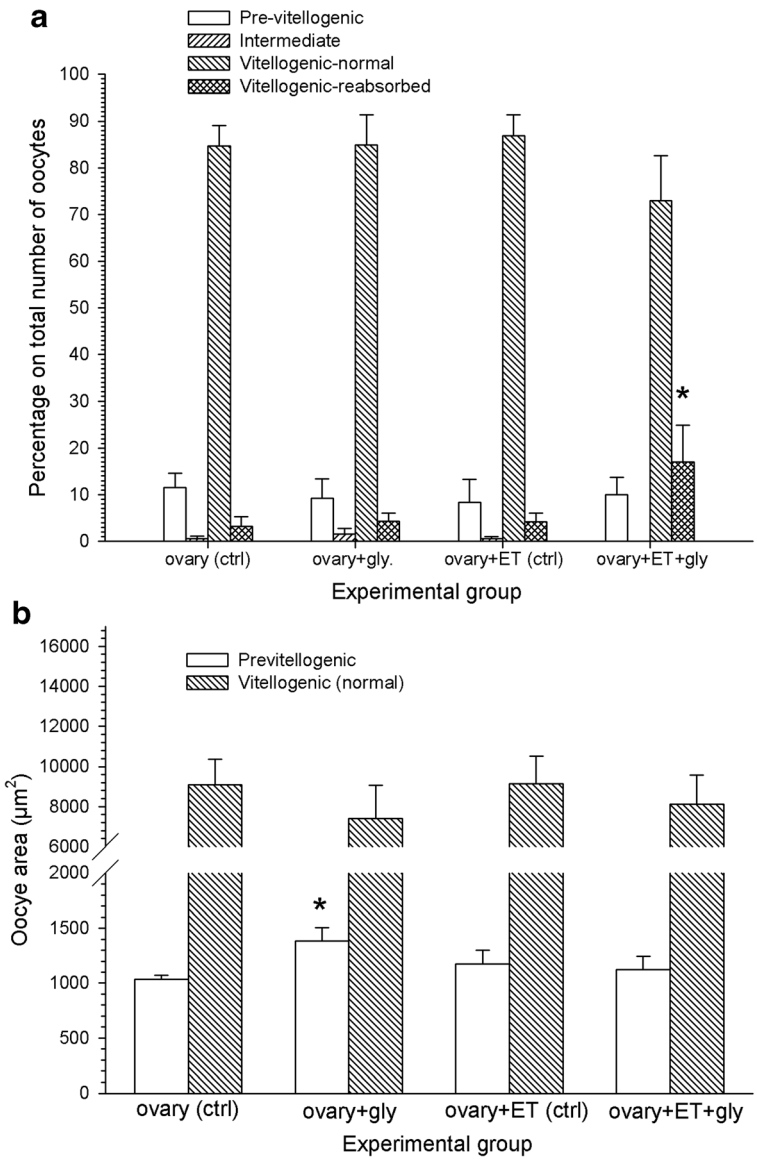

Fig. 4 **a** Proportion of oocyte type and **b** relative area of oocytes in the ovary (mean ± SE), from the in vitro experiments. ET: eyestalk tissue; Ctrl: control; Gly: glyphosate at 0.2 mg/L. Number of females (Nf) is indicated in Table 2. Asterisks indicate significant differences ($p < 0.05$) with respect to control. Since very few intermediate oocytes were observed, these were not considered for the area calculation

fact, GIH belongs to the CHH-family of peptides, and CHH (crustacean hyperglycemia hormone) have been proposed as the stress hormone of crustacean (Chang et al. 1999); its multifunctional role, including some effects on the ovary of some crustaceans, has been reported (Fanjul-Moles 2006; De Kleijn et al. 1998). Therefore, it should not be discarded that an augmented secretion of CHH from the eyestalks (or eventually other sites), expectable in a stressful situation, can impact on the ovary, leading to oocyte reabsorption.

On the other hand, the increment in the area of previtellogenic oocytes was observed in vivo, by effect of the assayed compounds, specifically by 1 mg/L of pure glyphosate. Moreover, this effect was also observed in vitro when glyphosate was added at 0.2 mg/L to the vials containing only ovarian pieces. This result is suggesting that glyphosate could stimulate the secretion of any stimulating hormone produced by the ovary, such as some kind of ovarian steroid (Charmantier et al. 1997; Nagaraju 2011), or could be acting as a steroidal agonist. Glyphosate could also bind to the ovary receptors of any other stimulating hormone of ovarian growth, acting as agonist. Among these hormones, methyl

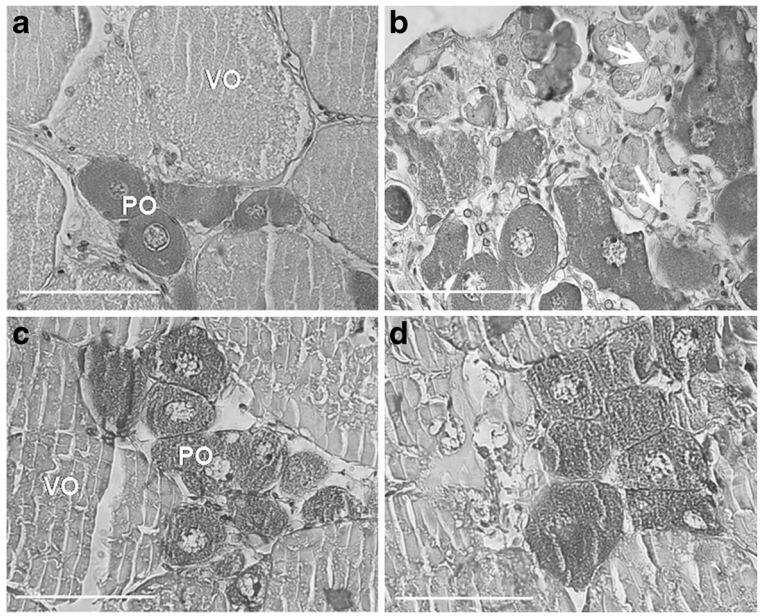

Fig. 5 Histological sections of the ovary, from both the in vivo (**a** control, **b** exposed to 1 mg/L of glyphosate) and the in vitro (**c** control, **d** exposed to 0.2 mg/L of glyphosate) experiments. Arrows indicate follicular cells participating in the reabsorption of vitellogenic oocytes (VO). A bigger size of previtellogenic oocytes (PO) can be observed in ovaries from the glyphosate treatments, compared to controls. Scale bars = 50 μm

farnesoate, secreted by the mandibular organ and/or the gonad stimulating hormone secreted by the thoracic ganglion (Nagaraju 2011; Charmantier et al. 1997), should be considered; to this respect, an interference of glyphosate with the nervous system of fish has been recently reported (Roy et al. 2016). Finally, the fact that the increment in the area of previtellogenic oocytes had not been seen in the in vitro preparation containing both ovary and eyestalk tissue was coherent with the presumably increase in the GIH secretion from the eyestalk by effect of glyphosate, as discussed above, which could counteract the stimulating effect of glyphosate on ovarian growth during the short-term in vitro assay.

5 Conclusions

In summary, glyphosate was able to produce, at relatively low concentrations, several harmful effects on adult female crabs during the pre-reproductive period. Firstly, the weight gain of adults was reduced in vivo by effect of pure glyphosate, at a concentration as low as 0.02 mg/L. In the ovary, the exposure to glyphosate caused, both in vivo (at 1 mg/L) and in vitro (at 0.2 mg/L), a significant reabsorption of vitellogenic oocytes, together with an increase in the area of previtellogenic oocytes. The in vitro experiments suggest that the active ingredient glyphosate could be causing some imbalances in the endocrine control of ovarian growth.

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