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EFFECTS OF GLYPHOSATE ON EGG INCUBATION, LARVAE HATCHING, AND OVARIAN REMATURATION IN THE ESTUARINE CRAB *NEOHELICE GRANULATA*

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Abstract: Ovigerous females of the estuarine crab (*Neohelice granulate*) were exposed to both pure glyphosate (2.5 mg/L and 5 mg/L) and a glyphosate formulation (Roundup Ultramax, containing glyphosate at 2.5 mg/L acid equivalent). At the end of the egg incubation period, a significant reduction in the number of hatched larvae was seen as a result of Roundup exposure. Additionally, several larvae abnormalities were seen in both pure glyphosate (2.5 mg/L) and Roundup treatments, such as hydropsy and hypopigmented eyes, and atrophied eyes were observed in the Roundup treatment. To evaluate the effect of the herbicide on ovarian rematuration, females remained exposed for 32 d. Pure glyphosate at 2.5 mg/L stimulated ovarian maturation over control levels, mainly in terms of a higher gonadosomatic index and a higher percentage of vitellogenic oocytes. A plausible hypothesis to be tested in further experiments is that exposure to glyphosate disrupts the hormonal system controlling reproduction. *Environ Toxicol Chem* 2014;33:1879–1884. © 2014 SETAC

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INTRODUCTION

Glyphosate (*N*-[phosphonomethyl]glycine) is currently among the herbicides more intensively applied on several crops in Argentina [1,2]. This is a nonselective, systemic herbicide that reduces plant growth by acting as a competitive 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 [3,4]. One of the main commercial formulations of this herbicide is Roundup (Monsanto). Water concentrations of glyphosate reported in the United States were relatively high, 2.7 mg (acid equivalent [a.e.])/L [5] and 2.8 mg (a.e.)/L [6]. Furthermore, up to 7.6 mg (a.e.)/L has been reported in Australia [7]. In Argentina, very scarce information about glyphosate environmental levels has been published, with reported values between 0.1 mg/L and 0.7 mg/L in water and between 0.5 mg/kg and 5 mg/kg in sediments [8].

Crustaceans represent a group of invertebrates widely distributed in marine, estuarial, and freshwater environments. In Argentina, the species *Neohelice* (=*Chasmagnathus*) *granulata* (Decapoda, Brachyura) 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. This species reproduces during the spring and summer. Toward the end of the egg incubation period, ovigerous females migrate offshore to allow hatching of larvae and subsequent larval development [9]. The megalopa stage returns to the coast to molt into the first juvenile instar, completing their life cycle. Both larvae and adults of several fish species are predators of larval and adult crabs, respectively [10,11].

Several rivers and channels that cross extensive agricultural areas and reach Samborombón Bay carry a heavy charge of pesticides, including herbicides [12]. Moreover, glyphosate is more intensively applied to both soybean and corn crops during summer [1], the reproductive period of *N. granulata*. Because of their well-known biology and their potential use as a sentinel species, *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 larval hatching [13–17]. However, relatively few studies about the disrupting effects of pesticides on the hormonal control of crustacean reproduction have been published [18,19]. There is only scant literature about the possible role of glyphosate as an endocrine disrupter compound. For example, at environmental concentrations, glyphosate has either antiandrogenic or anti-estrogenic effects, therefore acting as an endocrine disruptor compound of hormonal signaling for reproduction [20].

The present study aimed to evaluate the effects of glyphosate (both pure and formulated) on developing embryos, hatched larvae, and ovarian rematuration of *N. granulata* during the reproductive period.

MATERIALS AND METHODS

Ovigerous *N. granulata* females carrying immature eggs were randomly collected (13 October 2012) at the southern edge of Samborombón Bay, a nonpolluted area at the mouth of the Rio de la Plata estuary, Argentina. Once in the laboratory, they were acclimated for 12 h at the same environmental conditions and water quality used later for bioassays. All bioassays were conducted in semistatic conditions according to the standard procedures recommended by the American Public Health Association [21].

Stock solutions of glyphosate (as acid, 99.8% purity; Sigma) and Roundup Ultramax (as soluble granules, 67.9% w/w of glyphosate a.e.; Monsanto) were prepared weekly by dissolving the appropriate amount of the chemicals in distilled water. Small aliquots from these stock solutions were added to the test

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recipients with the appropriate dilution water, as stated below. The nominal concentrations assayed for pure glyphosate were 2.5 mg/L and 5 mg/L, whereas 2.5 mg/L was used for the Roundup Ultramax treatment. These sublethal concentrations were chosen according to the results of a preliminary range-finding test, carried out according to the guidelines given by the American Public Health Association [21]. Prior to the beginning of the assay, replicated water samples (15 mL) were taken at 0 h and 72 h, the period for water replacement in all test containers, to validate nominal concentrations. Glyphosate concentration was determined with ion-exchange chromatography, using a Dionex DX-100 chromatograph with a conductivity detector and a 25-mL sample loop [22]. A Dionex AS-4 was used as an analytical chromatographic column with an experimental error below 5%. A mixture of $NaOH/Na_2CO_3$ 4 mM/9 mM was chosen as eluent with a flow rate of 2 mL/min.

Effects on hatching

Eleven ovigerous females were randomly assigned to each treatment and control, and each female was placed in a glass container filled with 500 mL of saline water prepared by diluting artificial seawater salts (Tetra Marine Salt Pro) in dechlorinated tap water (hardness 80 mg/L as equivalents of $CaCO_3$, final salinity 30‰, pH 8.0 ± 0.5) provided with constant aeration. The solution of each container was completely replaced every 72 h. Females were not fed during the assay, and they were checked daily for mortality, egg loss, and hatching larvae. A temperature of $25 \pm 1\ ^{\circ}C$ and a photoperiod of 14:10 h (light: dark) were maintained throughout. The exposure period comprised an overall mean of 9.86 ± 0.57 d ($n = 44$), from the beginning of the assay until larval hatching.

After hatching, 2 samples of 10 mL each were taken from each container, stirred to homogenize larvae distribution, and fixed in 5% formalin. The total estimated mean number of hatched larvae was calculated from 2 random samples taken in relation to the total volume of water in the container. To determine the proportion of morphological abnormalities in each spawning, a random subsample of 50 larvae was examined under a stereomicroscope, and the incidence of each abnormality detected was then registered.

Effects on ovarian rematuration

Posthatching, females remained exposed to the same treatments until day 32, counting from the beginning of the assay. They were maintained under the same experimental conditions described in *Effects on hatching* but fed pellets prepared in the laboratory (an amount equivalent to 5% of body mass, twice per week), supplemented with *Elodea* sp. fresh leaves ad libitum. At the end of the assay, females were weighed to determine body weight, and both the ovaries and the hepatopancreas were dissected and weighed to determine the gonadosomatic (GSI) and hepatosomatic (HSI) indexes as GSI or HSI = (gonad wet wt or hepatopancreas wet wt/body wt) × 100.

Ovaries were weighed and 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. For each animal, a representative section of the ovary was analyzed to determine both the relative proportions of normal and abnormal oocytes and oocyte area. Previtellogenic, intermediate, and vitellogenic oocytes were characterized according to their size and degree of basophilia. To assess the proportions of normal and abnormal oocytes, a grid of 100 points, mounted on an 8× ocular lens, was used in combination with a 40× objective lens. At least 3 ovarian sections from each animal were examined by this procedure. For oocyte area, both major and minor diameters of the oocytes showing their nuclei were estimated by means of a micrometric ocular lens, calibrated against a Leitz Wetzlar plate with 1/100 mm spacing, to calculate the oocyte area as $(\pi/4) \times$ major diameter × minor diameter [23].

Statistical analysis

A one-way analysis of variance followed by least significant difference multiple comparisons was used for testing differences between experimental groups concerning incubation time, number of hatched larvae, proportion of each abnormality, proportion of each oocyte type, and oocyte area. Logarithmic or angular transformation of data was used when homogeneity of variances was not confirmed in raw data by the Bartlett test. Proportions of both surviving females and females with egg loss were compared between experimental groups by the Fisher exact test [24].

RESULTS

Measured concentrations of both pure glyphosate and Roundup were relatively close to the nominal concentration ($r^2 = 0.988$ and 0.954 for 0 h and 72 h, respectively; Figure 1).

Effects on hatching

Ovigerous females exposed to glyphosate did not experience mortality during the egg incubation period (Table 1). In addition, no significant ($p > 0.05$) differences were observed among mean body weight of females (overall mean 9.85 ± 0.43 g). Because no loss of eggs was verified in any case, 100% of females had normal hatching. No significant differences ($p > 0.05$) were observed in incubation time, although a significant ($p < 0.05$) reduction in the number of hatched larvae per female was observed in the Roundup treatment compared with control (Table 1).

Morphological abnormalities observed in larvae hatched from ovigerous females exposed to glyphosate during the egg incubation period were as follows (Figure 2): hydropsy, related to an abnormal hydration of tissues, especially in the cephalotorax; atrophy of spines and setae, probably underdeveloped, especially in the maxillipeds; atrophy of eyes, which showed an abnormal contour; and hypopigmented eyes, because

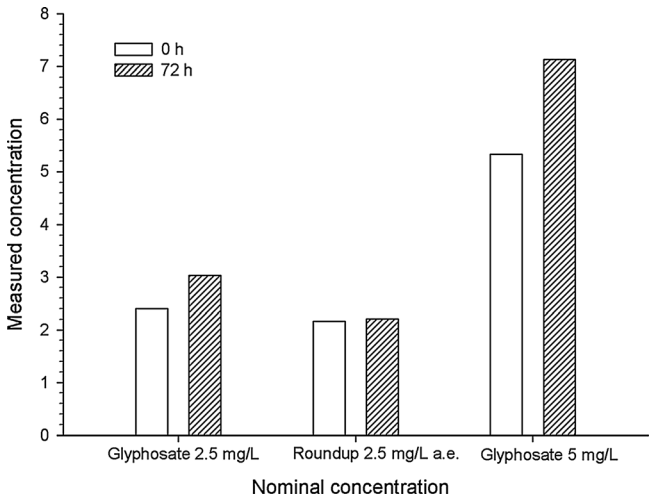

Figure 1. Nominal concentrations versus concentrations measured at 0 h and 72 h of the water replacement period.

Table 1. Percentage of hatching females, incubation time, and number of hatched larvae per female for each treatment

Glyphosate nominal concentration (mg/L a.e.)	No. of females	Hatching females (%)	Incubation time (d)[a]	No. of hatched larvae per female[a]
Control	11	100.00	9.72 ± 1.28	$33\,745 \pm 4969$
Glyphosate, 2.5	11	100.00	11.27 ± 1.34	$26\,739 \pm 5519$
Glyphosate, 5	11	100.00	9.18 ± 0.76	$25\,472 \pm 4472$
Roundup, 2.5	11	100.00	9.27 ± 1.11	$15\,448 \pm 2036^{*}$

[a]Mean ± standard error.
*Significant difference ($p < 0.05$) with respect to control.
a.e. = acid equivalent.

of loss of screening pigments in ommatidia. The incidence of these abnormalities is shown in Figure 3. Compared with the control group, a significantly ($p < 0.05$) higher incidence of both hydropsy and hypopigmented eyes was observed for both glyphosate and Roundup groups (2.5 mg/L of glyphosate a.e. in both cases). Moreover, a higher incidence of atrophied eyes was observed in the Roundup treatment.

Effects on ovarian rematuration

No significant mortality of females exposed to glyphosate during the 32 d of the ovarian rematuration assay was seen when compared with the control group, for which mortality reached 27% at the end of the assay.

Both GSI and HSI, determined at the end of the 32-d rematuration assay, are shown in Figure 4. Whereas a higher HSI was observed only for the lower glyphosate concentration compared with the control, a significantly higher GSI value was observed in the group exposed to pure glyphosate at both concentrations assayed. No significant ($p > 0.05$) differences were seen in the area of any type of normal oocytes whose mean area values were $1158.62 \pm 42.36\ \mu m^2$, $4013.50 \pm 286.98\ \mu m^2$, and $10\,322.87 \pm 1614.91\ \mu m^2$ for previtellogenic, intermediate, and vitellogenic oocytes, respectively. These types of oocytes are shown in Figure 5, and percentages of normal and reabsorbed oocytes are shown in Table 2. Significant ($p < 0.05$) differences in the percentage of normal oocytes were only found between control and glyphosate at 2.5 mg/L, indicating a lower percentage of intermediate and a higher percentage of vitellogenic oocytes with exposure to the herbicide. Although glyphosate increased the percentage of reabsorbed oocytes (particularly the vitellogenic ones in Roundup treatment, Figure 4), no significant ($p > 0.05$) differences with respect to control were detected.

DISCUSSION

Glyphosate concentrations used in the present study were similar to some environmental levels reported worldwide, which range from 2.7 mg/L (United States) to 7.6 mg/L (Australia) [5,7]. Although some of these examples could be considered as worst cases, this could be precisely the case of runoff water from crop fields (especially soy) loading glyphosate and discharging in water bodies where invertebrates live and reproduce. Although in Argentina, as mentioned in the *Introduction*, the scarce information available indicates relatively low glyphosate environmental levels (0.1–0.7 mg/L [8]), higher levels are actually expected in most Argentinean water bodies directly subjected to glyphosate contamination. This presumption is based on the several glyphosate formulations currently applied to an extension of about 20 million hectares of soybean [2], a situation comparable to that of other soy-producing countries, such as the United States.

At the end of the egg incubation period, all *N. granulata* ovigerous females were alive, providing evidence that assayed concentrations were sublethal for the adults, at least during the approximately 10 d of the incubation period. However, at the end

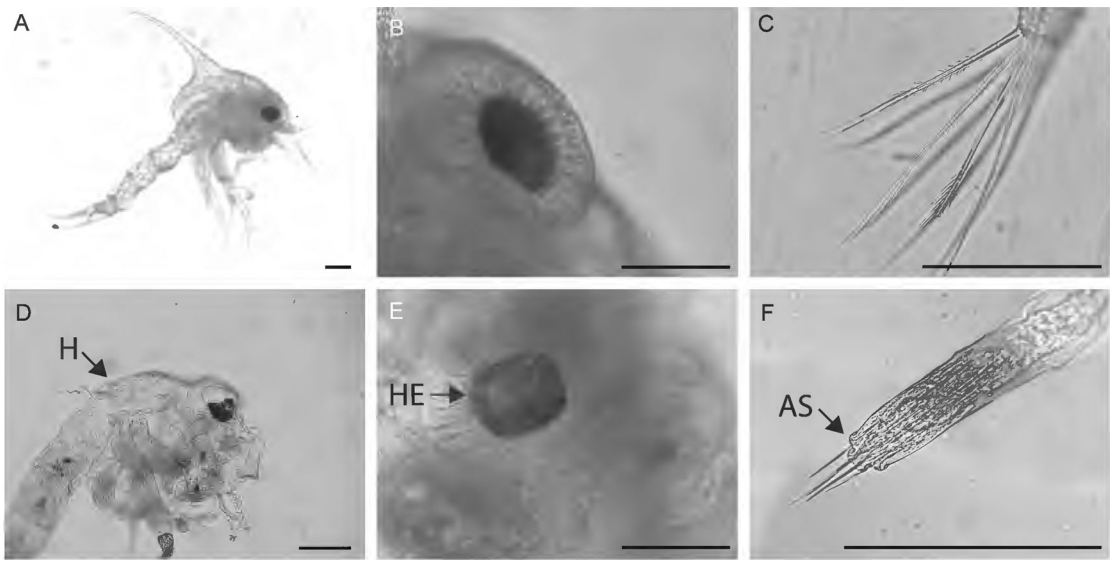

Figure 2. Abnormalities seen in larvae hatched from ovigerous females of *Neohelice granulata* exposed to glyphosate. (**A–C**) Control, (**D–F**) exposed to pure glyphosate. HE = hypopigmented eye; H = hydropsy; AS = atrophied maxilliped setae. Scale bar = 100 μm.

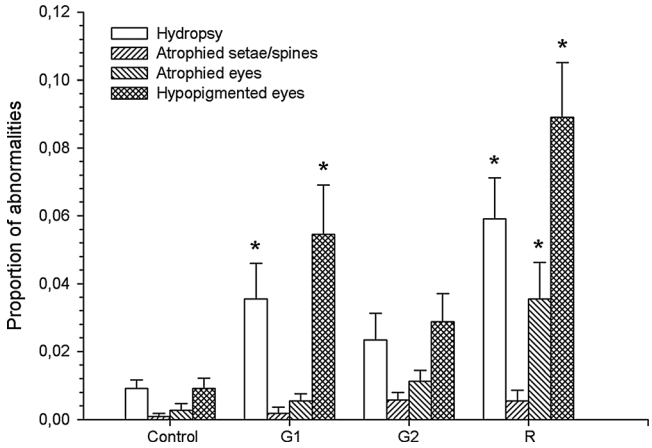

Figure 3. Morphological abnormalities in larvae hatched from ovigerous females exposed to either glyphosate (G) or Roundup (R). Mean ± standard errors are indicated. G1 = 2.5 mg/L acid equivalent (a.e.); G2 = 5 mg/L a.e.; R = 2.5 mg/L a.e. *Significant difference ($p < 0.05$) with respect to control.

of the 32-d rematuration assay, some mortality of females was observed, averaging 9% in the glyphosate treatments, which did not differ from control. Because a significantly lower number of hatched larvae per female was detected in the Roundup Ultramax treatment, a clear embryonic mortality was associated with this formulation, which contained a glyphosate concentration of 2.5 mg/L. Also taking into account that pure glyphosate, at the same concentration, did not significantly reduce the number of hatched larvae, these results indicate that Roundup compounds other than glyphosate may be responsible for the embryonic mortality. These results agree with those concerning enhanced lethal toxicity of glyphosate formulations on crustacean species compared with glyphosate alone [25,26]. In the species under study, a decreased number of hatched larvae have been reported with exposure to the pesticide parathion and 2,4-dichlorophenoxyacetic acid [13] and to the heavy metals cadmium and copper [14,15].

In the present study, several abnormalities were recognized in larvae hatched from ovigerous females exposed to glyphosate. Although similar abnormalities had been found in this species when exposed to either different pesticides [13] or several heavy metals [14,15], the relative incidence of the abnormalities

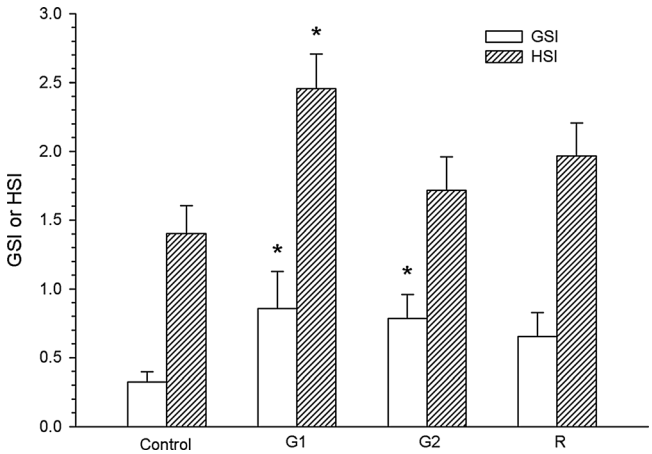

Figure 4. Gonadosomatic index (GSI) and hepatosomatic index (HSI) for each treatment at the end of the 32-d assay. G1 = 2.5 mg/L acid equivalent (a.e.); G2 = 5 mg/L a.e.; R = 2.5 mg/L a.e. Mean ± standard errors are indicated. *Significant difference ($p < 0.05$) with respect to control.

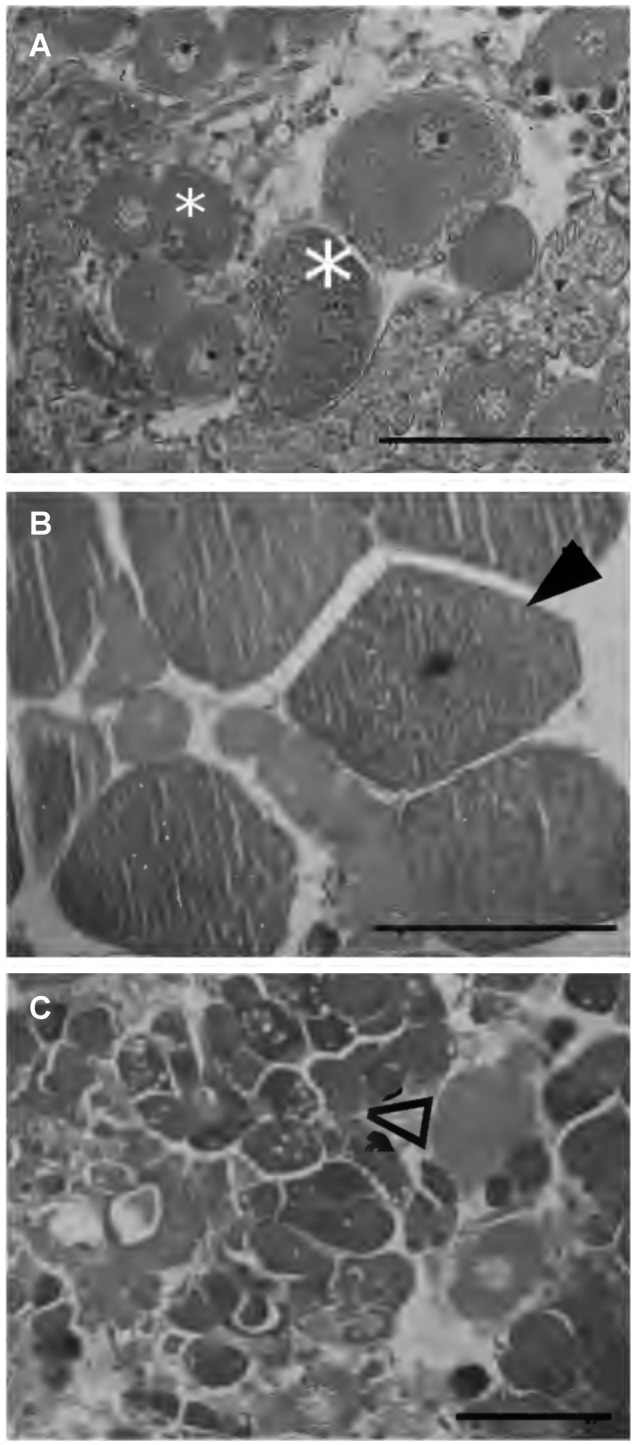

Figure 5. Histological sections of ovary of *Neohelice granulata*. (**A**) Control, with only previtellogenic (small asterisk) and intermediate (large asterisk) oocytes. (**B**) Glyphosate at 2.5 mg/L, with vitellogenic oocytes (solid arrowhead). (**C**) Roundup, at 2.5 mg/L, showing reabsorbed, vitellogenic oocytes (open arrowhead). Scale bar = 50 μm.

detected in the present study showed a particular pattern. In this respect, the abnormalities that showed a higher incidence were related to the eyes (atrophy and hypopigmentation). Severe atrophy of the eyes, but not hypopigmentation, has been reported with exposure to parathion, a neurotoxic xenobiotic [13], whereas hypopigmented, but not atrophied, eyes were reported after exposure to some heavy metals, such as copper, zinc, and mercury [15–17]. In contrast, hydropsy and atrophy of spines

Table 2. Percentage of different types of oocyte and oocyte size for each treatment at the end of the 32-d assay

Glyphosate nominal concentration (mg/L a.e.)	Previtellogenic oocytes		Intermediate oocytes	Vitellogenic oocytes	
	% Normal	% Reabsorbed	% Normal	% Normal	% Reabsorbed
Control, 0	57.64	0.00	27.51	0.00	14.86
Glyphosate, 2.5	38.98	3.09	0.00*	37.24*	20.69
Glyphosate, 5	44.72	2.46	18.01	28.34	6.48
Roundup, 2.5	51.10	2.70	9.26	9.22	27.71

*Significant difference ($p < 0.05$) with respect to control.
a.e. = acid equivalent.

and setae seem to be clearly unspecific pathologies, because they were reported by all of the studies mentioned above.

Genotoxic effects (induction of micronuclei) have been described in fish exposed to some glyphosate-based formulations [27]. Several genotoxic and developmental effects of glyphosate and Roundup were also reported in South American caimans [28]. In the present study, although atrophy of the eyes was observed only in the Roundup formulation treatment, hypopigmented eyes were observed in both Roundup and pure glyphosate groups at equal concentrations of acid equivalents. This result clearly indicates that some of the abnormalities detected, particularly hydropsy and hypopigmented eyes, are caused by glyphosate itself. Mottier et al. [29] have reported several embryonic abnormalities and even arrested development in oysters exposed to both pure glyphosate and some Roundup formulations, which showed the highest toxicity.

Several effects of glyphosate on ovarian rematuration were observed. A GSI higher than that of the control was observed in all glyphosate treatments; the increase was statistically significant only with pure glyphosate, however, and not with Roundup. This result could be related to the increased oocyte reabsorption (28%) caused by Roundup, particularly for the vitellogenic oocytes, although this reabsorption was masked by the relatively high reabsorption (14%) that took place in control females. The high reabsorption rate in controls is expected as a result of oocytes not extruded in the spawning that occurred 1 mo previously. However, the augmented reabsorption of vitellogenic oocytes caused by Roundup could be related to an unspecific response against this herbicide as a stressor. In this way, the reabsorption of ovarian lipovitellins would allow the organism to relocate the energy needed to cope with a stressful situation [30].

Moreover, the lower degree of maturation shown by control ovaries in comparison with ovaries belonging to females exposed to glyphosate suggests a possible effect of glyphosate as an endocrine disruptor. In control females, most of the normal oocytes (58%) were previtellogenic and a smaller percentage (28%) were intermediate; no vitellogenic, normal oocytes were observed. In contrast, at 2.5 mg/L of glyphosate, the percentage of normal vitellogenic oocytes (37%) was significantly higher than control levels, in close correlation with the augmented GSI and HSI observed at this glyphosate concentration. Although not significantly different from control, the same tendency was also observed at the highest glyphosate concentration. It is possible that a high glyphosate concentration can induce a stronger detoxification response against xenobiotics compared with a lower concentration, as reported for other pesticides [18]. Concerning Roundup, the observed low percentage of normal vitellogenic oocytes seems to be related to the high percentage of vitellogenic oocyte reabsorption caused by this formulation, as mentioned above.

In a previous study on *N. granulata* females exposed to parathion, a larger size of both previtellogenic and vitellogenic oocytes was seen [23]; this effect could be related to interference with the secretion of the gonad-inhibiting hormone at the eyestalks [18]. From an ecotoxicological point of view, the acceleration of ovarian maturation could lead to spawning out of the normal reproductive season, a clear disadvantage for reproductive success. Concerning fish species, no changes in fecundity or GSI were observed in adult rainbow trout treated with the isopropylamine salt or Roundup up to 2.0 mg/L [31]. However, decreased levels of estradiol together with a higher value of the HSI were reported in fish exposed to 3.6 mg/L of glyphosate [32].

In summary, several sublethal effects of glyphosate, both pure and formulated at a concentration of 2.5 mg/L, were observed in *N. granulata* females. These effects mainly consisted of a reduction of hatched larvae caused by Roundup, some abnormalities (hydropsy, abnormal eyes) in larvae hatched in both pure glyphosate and Roundup as well as stimulation of ovarian maturation by pure glyphosate, both in terms of a higher GSI and a higher percentage of vitellogenic oocytes.

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