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Antioxidant and Histopathological Effects of Paraquat and Fluroxypyr Herbicides on the Apple Snail *Pomacea canaliculata* (Lamarck, 1822)

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Abstract: Argentina is among the top consumers of herbicides, yet studies on their environmental and health impact remain scarce. This work aimed to evaluate the effects of herbicide exposure on *Pomacea canaliculata* as potential biomarkers of contamination. Specifically, we investigated whether paraquat (Pq) and fluroxypyr (Fx) alter enzymatic antioxidant defenses in tissues following acute exposure and induce histological modifications in the digestive gland (DG), particularly in symbiotic corpuscles, after chronic exposure. The nominal no-observed-effect concentration on lethality ($NOEC_L$) values were 3.62 µg/g dry mass (DM) for Pq and 10.42 µg/g DM for Fx. After acute exposure, superoxide dismutase activity decreased in the DG but increased in the kidney for both herbicides. Catalase activity decreased in the gills but increased in the kidneys of exposed snails, while glutathione-S-transferase activity increased in the DG and kidney after Pq exposure. Following chronic exposure (Pq: 1.45 µg/g DM; Fx: 6.94 µg/g DM), epithelial thickening and vacuolization were observed in Fx-exposed snails. Morphometric analysis of the DG showed that Pq reduced the epithelial occupancy of the symbiont's vegetative form while increasing its cystic form. These findings indicate that both herbicides impact antioxidant defenses, DG function and host–symbiont interactions, reinforcing the suitability of *P. canaliculata* as bioindicator organisms.

Keywords: aquatic contamination; agrochemicals; pesticides; oxidative stress; symbiosis; *Pomacea canaliculata*

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1. Introduction

Apple snails (Ampullariidae) represent a diverse family of gastropods widely distributed in freshwater ecosystems worldwide [1–3]. Neotropical ampullariids include species from the genera *Pomacea*, *Asolene*, *Felipponea*, and *Marisa* [2]. Among these, *Pomacea canaliculata* (Lamarck, 1822) is the most extensively studied species due to its high invasiveness and broad global distribution [4,5]. This species exhibits remarkable physiological plasticity, enabling it to cope with diverse environmental stressors, including pH fluctuations, prolonged droughts, and challenging environmental conditions [2]. Such physiological adaptability contributes significantly to its invasive success [6–9]. For example, *P. canaliculata* employs a suite of behavioral, physiological, and molecular mechanisms

to reduce its metabolic rate and survive under hypometabolic conditions, such as those induced by hibernation or estivation, and it can rapidly reactivate its metabolism when favorable conditions return [7–9].

The diverse morphological and physiological adaptations of *P. canaliculata* align with its diet and feeding strategies [2,10,11]. Its digestive epithelium harbors intracellular symbiotic corpuscles (designated C and K), which secrete a protease into the digestive tract [11,12]. More recently, toxicological studies have suggested that these symbionts play a role in the accumulation of non-essential toxic elements and are directly affected by insecticides [13–17]. Arrighetti et al. [17] reported a significant increase in the percentage of digestive gland acinar area occupied by K corpuscles in snails exposed to cypermethrin at different sublethal concentrations, with a progressive rise observed over longer exposure durations. Notably, they also reported other histopathological alterations in the digestive gland, including hemocyte infiltration, and an increase in the number of "basophilic" cells (i.e., possibly pyramidal cells containing K corpuscles), epithelial atrophy, and acinar necrosis. These effects were particularly pronounced in snails exposed to the highest sublethal concentrations (25 and 100 µg/L) for 7 and 14 days [17].

The widespread global distribution of apple snails has substantial ecological and economic implications [18]. However, their frequent occurrence in aquatic environments, particularly in agricultural ecosystems, presents an opportunity to explore their potential as bioindicator organisms [19]. *Pomacea canaliculata* possesses several traits that make it a promising bioindicator of environmental pollution, including its large body and population size, amphibious lifestyle, semi-sessile behavior, high reproductive rate, and molecular adaptations, that enhance survival under adverse conditions [15].

Over the past decade, physiological and biochemical responses to pollutants have gained recognition as biomarkers in apple snails [19,20]. Studies have evidenced their capacity to accumulate non-essential toxic elements, such as mercury, arsenic, cadmium, uranium, and copper, primarily in detoxification organs such as the kidney and digestive gland [13–15,21]. Furthermore, exposure to organotin compounds such as tributyltin and triphenyltin has been shown to induce reproductive alterations and masculinization in adult females [22–24]. Additionally, histopathological changes in *P. canaliculata* tissues have been reported following exposure to contaminants such as pesticides (e.g., cypermethrin) and heavy metals [14,16,17]. These effects include tissue damage and enzymatic disruptions, such as acetylcholinesterase inhibition [25], a widely used biomarker for pesticide exposure, particularly to organophosphates. The release of xenobiotics or their active metabolites into aquatic environments exacerbates pollution and may indirectly affect predators of these snails. Collectively, these characteristics position *P. canaliculata* as a key species for monitoring anthropogenic pollutants and assessing ecotoxicological effects in freshwater and agricultural ecosystems.

This work aimed to evaluate the effects of herbicide exposure on *P. canaliculata* as potential biomarkers of contamination. Specifically, we investigated whether paraquat (Pq) and fluroxypyr (Fx) alter enzymatic antioxidant defenses in tissues following acute exposure and induce histological modifications in the digestive gland, particularly in symbiotic corpuscles, after chronic exposure.

2. Results

2.1. Herbicide Toxicity in P. canaliculata

No mortality was observed in snails exposed to the vehicle solution. The no-observed-effect concentration on lethality ($NOEC_L$) was 10.42 µg/g drained mass (DM) for Fx and 3.62 µg/g DM for Pq, indicating that Pq is more toxic to *P. canaliculata* than Fx.

2.2. Antioxidant Enzyme Activities

The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) were measured in the digestive gland (DG), kidney, and gill of herbicide-exposed and non-exposed (control) snails (Figure 1).

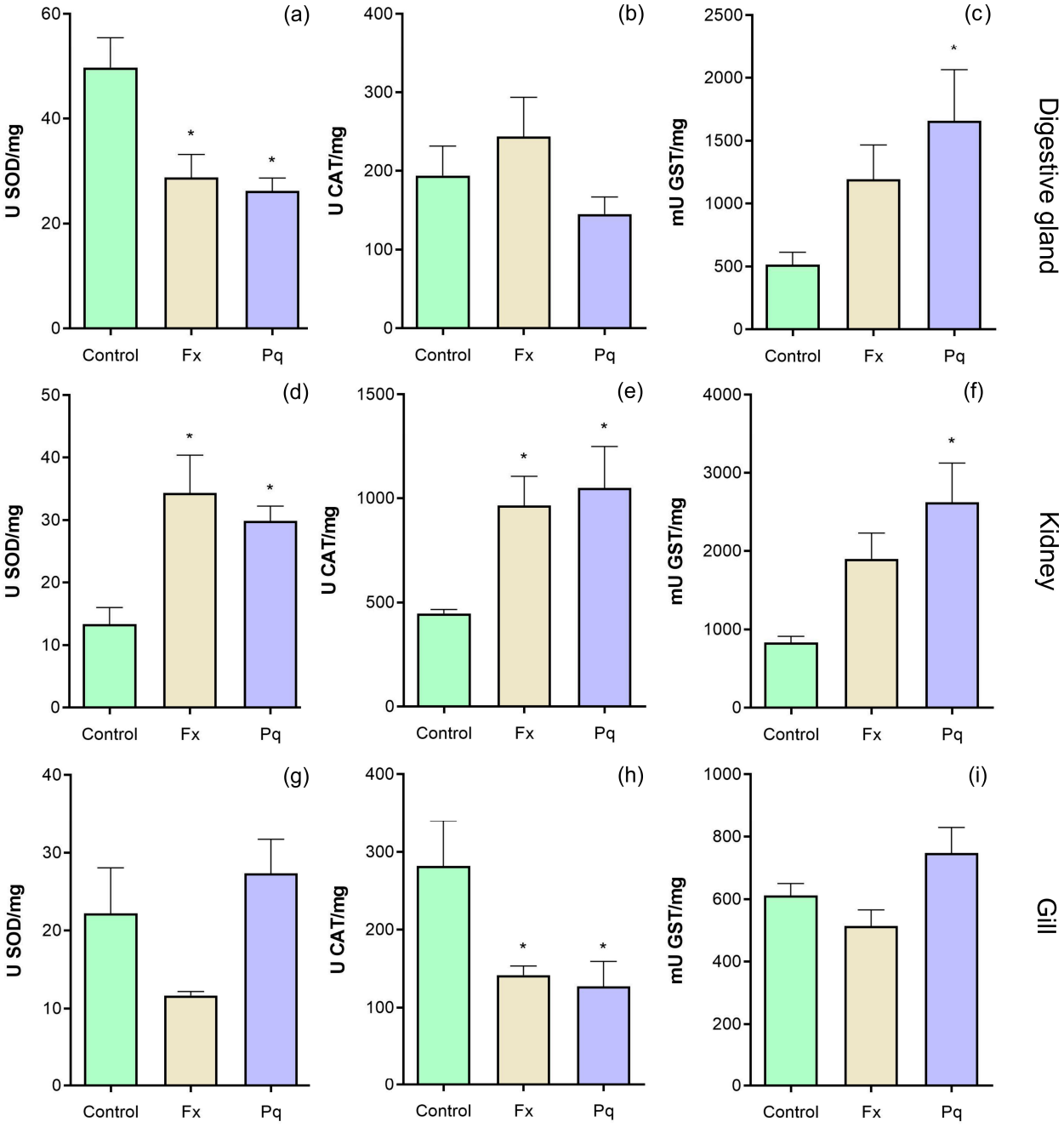


Figure 1. Antioxidant enzyme activities, superoxide dismutase (SOD; (**a**,**d**,**g**)), catalase (CAT; (**b**,**e**,**h**)) and glutathione S-transferase (GST; (**c**,**f**,**i**)), in the digestive gland, kidney, and gill of *P. canaliculata* under control conditions and after exposure to fluroxypyr (Fx) or paraquat (Pq). * indicate significant differences between exposed groups and the control group (N = 5, mean ± SEM) (ANOVA, Dunnett's post hoc test, $p < 0.05$).

Compared to the control group, SOD activity significantly decreased in the DG of snails exposed to Fx (58%) and Pq (53%) (Figure 1a, Supplementary Table S1). In contrast, SOD activity more than doubled in the kidney of herbicide-exposed snails (Figure 1d,

Supplementary Table S1). No significant changes were observed in the gills (Figure 1g, Supplementary Table S1).

CAT activity remained unchanged in the DG (Figure 1b, Supplementary Table S1). However, it significantly increased in the kidneys of Fx- and Pq-exposed animals (Figure 1e, Supplementary Table S1), reaching 216% and 235% of control levels, respectively. Conversely, CAT activity in the gill was markedly reduced (Fx: 50%, Pq: 45%) (Figure 1h, Supplementary Table S1).

GST activity significantly increased in the DG and kidney of Pq-exposed snails, with no significant changes in the gills (Figure 1c,f,i, and Supplementary Table S1). In relative terms, GST activity rose to 322% in the DG and 315% in the kidney following Pq exposure, whereas Fx-exposed snails showed a more moderate increase (232%) in the DG and 228% in the kidney. In the gill, GST activity remained relatively stable, with a slight reduction in Fx-exposed individuals (−16%) and a moderate increase in the Pq group (22%).

2.3. Histological Changes in the Digestive Gland Induced by Herbicides

Trichrome-stained sections of control snails revealed a typical tubuloacinar structure in the digestive gland (Figure 2). As expected, adenomeres were composed primarily of columnar and pyramidal cells (Figure 2g,j). Secretory ducts were lined by ciliated columnar epithelium with scattered goblet cells, while storing cells, connective tissue, and hemocoelic spaces were also present (Figure 2a–c). Columnar cells were elongated, vacuolated, and contained Alcian blue–positive C symbiotic corpuscles (Figure 2g). Pyramidal cells exhibited a triangular morphology with purple cytoplasm and were frequently associated with K symbiotic corpuscles, which retained their naturally dark-brown pigmentation and remained unstained (Figure 2j, high magnification).

Chronic exposure to herbicides induced distinct histological alterations in the tubuloacinar structure of the digestive gland.

In Fx-exposed snails, columnar epithelial cells displayed reduced apical vacuolization, with eosinophilic granules occupying these spaces (Figure 2e,h). Additionally, Fx exposure led to a decrease in the number of pyramidal cells and a concomitant reduction in pigmented K corpuscles.

In Pq-exposed snails, columnar cells exhibited a reduction in C symbiotic corpuscles and an increase in vacuolization, characterized by Alcian blue-positive inner clumps (Figure 2c,f,i). However, pyramidal cells within the adenomeres appeared well preserved.

2.4. Symbiotic Occupancy of the Digestive Gland

Given the histological alterations observed in the adenomeres and symbiotic corpuscles, we quantified the symbiotic occupancy of the DG in herbicide-exposed snails. In control snails, C and K corpuscles were identified without staining by their characteristic greenish (CpsC) or dark-brown (CpsK) pigmentation, respectively (Figure 3a–c).

In control animals, symbiotic corpuscles occupied approximately 20% of the total adenomere area, with a relative proportion of 42% C corpuscles and 58% K corpuscles (Figure 3d). In Fx-exposed snails, total symbiotic occupancy showed a slight, non-significant reduction while maintaining the same C/K ratio as in controls (Figure 3d–f). Conversely, in Pq-exposed snails, total symbiotic occupancy remained stable (Figure 3d), but the relative proportion of C corpuscles in columnar cells significantly decreased, accompanied by a corresponding increase in K corpuscles associated with pyramidal cells (Figure 3e,f).

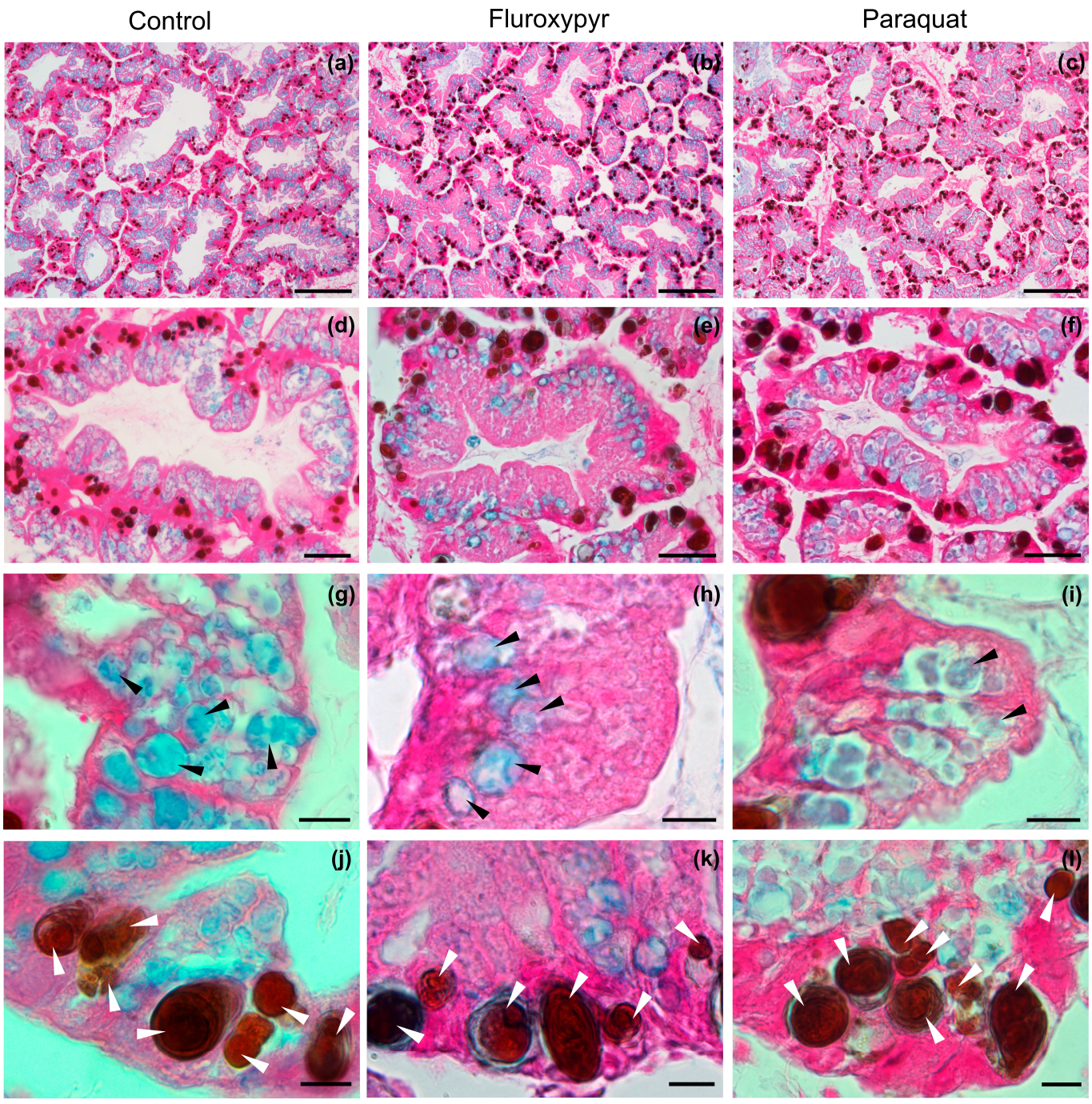


Figure 2. Histological analysis of the digestive gland in *P. canaliculata* under control conditions and after herbicide exposure. Panels (**a**–**c**) (scale bars: 100 μm) display adenomeres at low-magnification highlighting general acinar changes, including eosinophilic granule accumulation at the luminal edge of the acinar epithelium in Fluroxypyr-exposed snails (**b**). In contrast, the adenomere of Paraquat-exposed animals exhibits an increased presence of the cystic symbiotic form (**c**). Panels (**d**–**f**) (scale bars: 50 μm) provide higher-magnification of the adenomere, highlighting columnar cells containing CpsC with alcianophilic inner clumps and covers, and pyramidal cells containing brown multilamellar CpsK. Panels (**g**–**i**) show a detailed view of the columnar cell domes with CpsC (black arrowheads), while panels (**j**–**l**) (scale bars: 10 μm) present greater detail of the pyramidal cells with CpsK (white arrowheads).

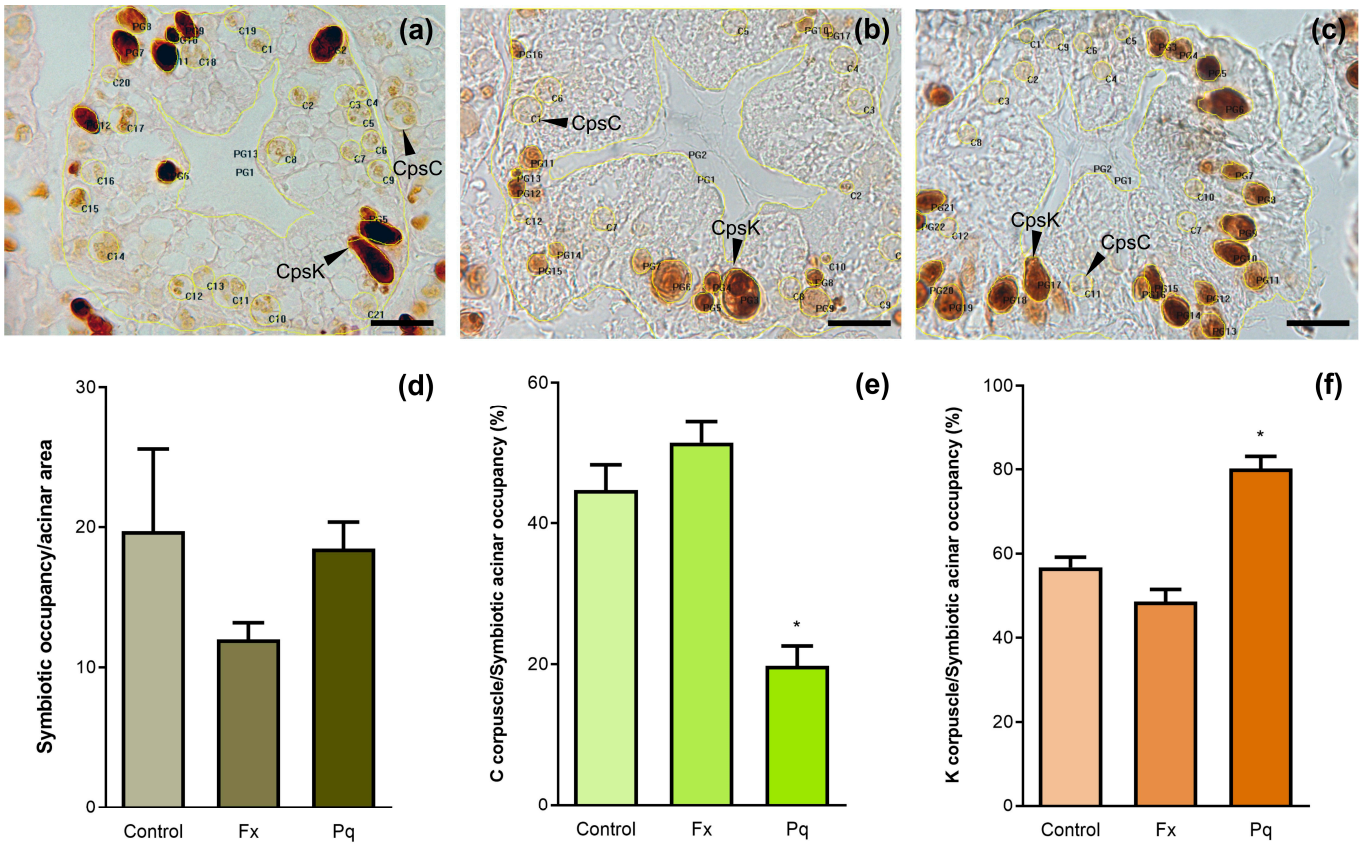


Figure 3. Morphometric analysis of the digestive gland in *P. canaliculata* under control conditions and after herbicide exposure. Images captured from the software (Image Pro Plus® 6.0, Media Cybernetics, Silver Spring, MA, USA) used for morphometric quantification of greenish vegetative (CpsC) and brown cystic (CpsK) forms of the intracellular symbiont (indicated with an arrowhead) in histological sections without staining where in control animals (**a**), exposed to fluroxypyr (**b**) and paraquat (**c**). Total symbiotic occupancy (**d**) expressed as percentage of the total epithelial area. Relative percentage of CpsC occupancy (**e**). Relative percentage of CpsK occupancy (**f**). * indicate significant differences in C or K corpuscle occupancy between herbicide-exposed snails and the control group (N = 3, mean ± SEM) (ANOVA, Dunnett's post hoc test, Pq-CpsC $p = 0.0025$; Pq-CpsK $p = 0.0017$).

3. Discussion

3.1. Enzymatic Antioxidant Response to Herbicides

The cellular antioxidant defense system plays a crucial role in maintaining homeostasis by regulating free radical levels and preventing damage to macromolecules [26]. Disruptions in these defense mechanisms in aquatic organisms can serve as sensitive and specific biomarkers of pollutant exposure [27]. Given that both herbicides, Pq and Fx, generate superoxide anions —highly reactive oxygen species capable of inducing lipid peroxidation—and other reactive species [28,29], the present study is the first to evaluate the tissue-specific responses of the antioxidant enzymes SOD, CAT, and GST in *P. canaliculata* specimens exposed to these compounds.

SOD is a metalloprotein that catalyzes the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide [30]. CAT detoxifies hydrogen peroxide by converting it into water, preventing cellular damage [31]. GST, primarily known for its role in xenobiotic detoxification [27], also contributes to cellular antioxidant defenses protecting DNA and lipids from peroxidation [32]. These enzymes have been widely recognized as components of the antioxidant defense system in *P. canaliculata*, in response to various

stressors, such as estivation, hibernation, exposure to toxic elements, and insecticides like cypermethrin [6–9,13,17,33].

The digestive cell–symbiont consortium is involved in a diverse array of physiological functions, including carotenoid metabolism, extracellular digestion of macronutrients, and accumulation or depuration of non-essential toxic elements [11,12,14,15,34–37]. Furthermore, the digestive gland shows changes in its redox balance, when exposed to environmental stressors, with documented modifications in the antioxidant defense system and the consequent oxidative damage [6–9,13,17]. In this work, the SOD activity decreased after exposure to either Pq (3.62 μg/g) or Fx (10.42 μg/g), as observed in the freshwater gastropod *Biomphalaria glabrata* (Say 1818), exposed to low concentrations of Pq for 48 h (0.5 mg/L [38]). This could be related to direct alteration in Pq on the SOD activity or, alternatively, general damage in different intracellular components. CAT activity remained without changes, as also observed in *B. glabrata* after Pq exposure [38]. Finally, GST activity from the digestive gland increased in Pq-exposed snails and remained without significant changes in Fx-exposed animals.

The kidney in *P. canaliculata* plays a vital role in osmoregulation, as the internal milieu of this species is hyperosmotic relative to its freshwater habitat [39]. Additionally, the kidney serves three key functions: (i) it contains uric acid-rich concretions, which may act as an antioxidant molecule against oxidative stress [7,40]; (ii) it accumulates and eliminates non-essential toxic elements, such as mercury, arsenic, cadmium, and uranium [12,14,15]; and (iii) it functions as an immunological barrier against pathogens [41]. In the present study, Pq exposure led to increased kidney antioxidant enzyme activity, while both SOD and CAT activities were elevated in Fx-exposed animals. These findings suggest that oxidative stress induced by these herbicides may activate shared regulatory pathways, potentially involving the FOXO3 transcription factor, a REDOX-sensitive regulator previously shown to protect tissues under environmental stress [7,42].

It has been proposed that the gill of the apple snail does not have a primary function in respiration, as the thickness of its epithelium might limit gas diffusion [43]. Instead, the gill likely complements the kidney function by regulating water uptake and ion balance [43]. Supporting this hypothesis, the gill epithelium exhibits features typical of an ion-transporting rather than a respiratory tissue and contains abundant mitochondria, which could provide energy for ion exchange [43]. Given this high mitochondrial density, the gills may require a robust antioxidant system to counteract free radicals generated by oxidative metabolism. However, after acute (7-day) exposure to both Pq and Fx, SOD and GST activities in the gill remained unchanged, whereas CAT activity decreased significantly. This decline in CAT activity could indicate oxidative damage to protein structures, impairing the enzyme's function.

Overall, our findings highlight tissue-specific physiological responses to herbicide exposure, with the most pronounced effects occurring in the DG and kidney, reinforcing the complexity of antioxidant defense mechanisms in *P. canaliculata*. Future studies should consider additional variables, such as the role of non-enzymatic antioxidants, including uric acid [40], reduced glutathione, and lipophilic molecules (e.g., carotenoids [34,36]), which may provide compensatory protection when enzymatic defenses are compromised by herbicide exposure.

3.2. Effects of Herbicides on Digestive Gland Histology and Its Symbiotic Corpsucles

In animals, several histological changes have been associated with oxidative stress, including the disruption of tight junctions in epithelial cells, which compromises barrier function [44], and the epithelial disorganization by cytoskeleton disassembly and protein carbonylation [45], among others. Histological changes in mollusks caused by environ-

mental contaminants have been proposed as valuable indicators of ecosystem health [46]. Numerous studies have focused on the digestive gland of mollusks, as this organ is a synapomorphy of the phylum. However, ampullariid gastropods stand out as ideal bioindicators due to their relatively large size in freshwater ecosystems and the presence of a pigmented prokaryotic symbiont within their digestive gland [2,12,37]. This symbiont is molecularly similar to cyanobacteria and chloroplasts, yet it retains characteristics typical of free-living organisms [2,12,37].

These symbionts, referred to as C and K pigmented corpuscles [47], have been proposed to be the vegetative and cystic form of the same organism [48] due to the presence of several intermediate forms between these morphotypes (Figure 1C from [48]). Both pigmented corpuscles are contained within large vesicles (Figures 3D and 5A from [48]), in two different types of glandular epithelial cells: C corpuscles are present in columnar cells, whereas K corpuscles are present in pyramidal cells (as described previously in Section 2.3). The symbiont has been identified as a potential indicator of environmental contamination due to its ability to accumulate and detoxify heavy metals and uranium [12,14,15,21]. Furthermore, Arriguetti et al. [16,17] proposed that morphological and histological changes in the digestive gland of *P. canaliculata* could serve as sensitive indicators of exposure to the pesticide cypermethrin. These authors reported changes in the percentage of acinar area occupied by K corpuscles [17] but misidentified the C corpuscles as lipofuscin granules [16]. In this study, we evaluate the hypothesis that both pigmented symbiotic corpuscles can act as sensitive indicators of exposure to Pq and Fx, demonstrating that the morphometric analysis of glandular occupation is a suitable biomarker of freshwater contamination.

In the field of bioremediation and pesticide detoxification, several studies have examined the response of Cyanobacteria to herbicide exposure. Notably, some cyanobacterial species, such as *Chlamydomonas reinhardtii* (Dangeard, 1888), accumulate and rapidly degrade Fx, a mechanism proposed as a potential tool for environmental remediation [49]. However, considering our findings, where total glandular symbiotic occupancy decreased in animals exposed to Fx while maintaining the ratio of C and K corpuscles compared to control snails, it is likely that the symbiont of *P. canaliculata* is unable to metabolize Fx.

In contrast to Fx, some cyanobacterial species are susceptible to the herbicide Pq. For instance, exposure to Pq has been shown to significantly reduce dry mass, chlorophyll *a* content, and phycocyanin levels in *Nostoc* sp. N1 and *Anabaena* sp. A1 [50]. Similarly, in *Microcystis aeruginosa* (Kutzing, 1846), Pq exposure induces the overproduction of reactive oxygen species, leading to morphological changes, cellular structure disruption, and cell death [51–53]. Here, we observed that Pq exposure in *P. canaliculata*'s digestive gland results in a decrease in C corpuscles and an increase in the K morphotype. This shift may be linked to alterations previously described in response to Pq exposure in other organisms [54], such as disruptions in the respiratory chain, changes in cell wall structure, and the induction of phototoxic and cytotoxic effects.

The morphological transition from the vegetative form to the cystic form, marked by the accumulation of multiple concentric layers of electron-dense material, may represent a tolerance mechanism to withstand the herbicide's adverse effects [47,48]. Further research on the impact of herbicides on the digestive gland and its symbiotic inhabitants is essential to fully understand the effects of pesticides on both macro- and microbiota in aquatic environments. An alternative interpretation is that the decreased glandular occupancy by C corpuscles was due to cell death mediated by ROS. In *Escherichia coli*, it has been demonstrated that, although these bacteria may survive the initial exposure to a stressor and the post-stress accumulation of ROS can lead to cell death [55], this may be due to the stimulation of a self-amplifying cycle of ROS accumulation that overwhelms cellular repair mechanisms for the primary damage [55]. In the present study, the decrease in SOD

activity could lead to increased superoxide production. This anion is highly reactive and is associated with lipid peroxidation and the release of polyunsaturated fatty acids—followed by the fragmentation of peroxides to generate aldehydes—which results in the loss of membrane integrity [56]. This occurs through alterations in membrane fluidity, ultimately leading to the inactivation of membrane-bound proteins [56]. The loss of membrane integrity in the vesicles containing the symbionts may render them more vulnerable to oxidative stress, potentially leading to their death, as has been observed in *E. coli*.

4. Materials and Methods

4.1. Animals

Pomacea canaliculata adult females (4–5 months old) were used in the experiments. The animals were reared from hatching to adulthood in the laboratory, and the original stock was derived from Rosedal Lake (Palermo, Buenos Aires, Argentina). Rearing conditions followed previously established protocols [9]. Briefly, animals were maintained under controlled environmental conditions: temperature at 24–26 °C, relative humidity at 80%, and a 16:8 h light–dark cycle. Snails were housed in plastic containers, filled with 5 L of tap water. The water was replaced three times per week, and the animals were fed daily with fresh lettuce (*Lactuca sativa* L., Sp. Pl., 1753).

4.2. Agrochemicals and Experimental Design

The herbicides used in this study were Gramoxone® Super (active ingredient: paraquat; chemical group: bipyridyl) and Tomahawk® Magan (active ingredient: fluroxypyr; chemical group: synthetic auxin). Herbicide concentrations are expressed as micrograms of Pq or Fx per gram of drained mass (DM).

Each experiment consisted of three treatment groups:

(a) Control group: Animals received an injection of the vehicle solution (1 µL/g DM), an isotonic solution mimicking the hemolymph composition (86 mmol/L NaCl, 1.8 mmol/L KCl, 2.1 mmol/L $CaCl_2$, 10 mmol/L HEPES [22]).

(b) Paraquat (Pq) group: Animals were injected with Gramoxone® Super (20 g/100 mL of 1,1′-dimethyl-4,4′-bipyridyl).

(c) Fluroxypyr (Fx) group: Animals were injected with Tomahawk® Magan (20 g/100 mL of 4-amino-3,5-dichloro-6-fluoro-2-pyridloxyacetic acid).

After relaxing the animals in 4 °C water (physical method) for 10 min, the solutions were administered via intramuscular injection into the foot, using a sterile 50 µL Hamilton glass syringe (Hamilton Co., Inc., Whittier, CA, USA).

4.3. Toxicity Assesment

4.3.1. Toxicity Response

To determine the lethal effects of Pq and Fx, increasing doses of each herbicide were injected into adult female *P. canaliculata* following the procedure described in Section 4.2. The snails were then maintained for 48 h under the laboratory-controlled conditions previously described (Section 4.1), with no water changes during the exposure period. Each toxicity assay included eight snails per treatment group. The experiment was terminated once at least one exposed individual died. The no-observed-effect concentration on lethality ($NOEC_L$) was defined as the highest tested concentration at which no mortality was observed (Figure 4a).

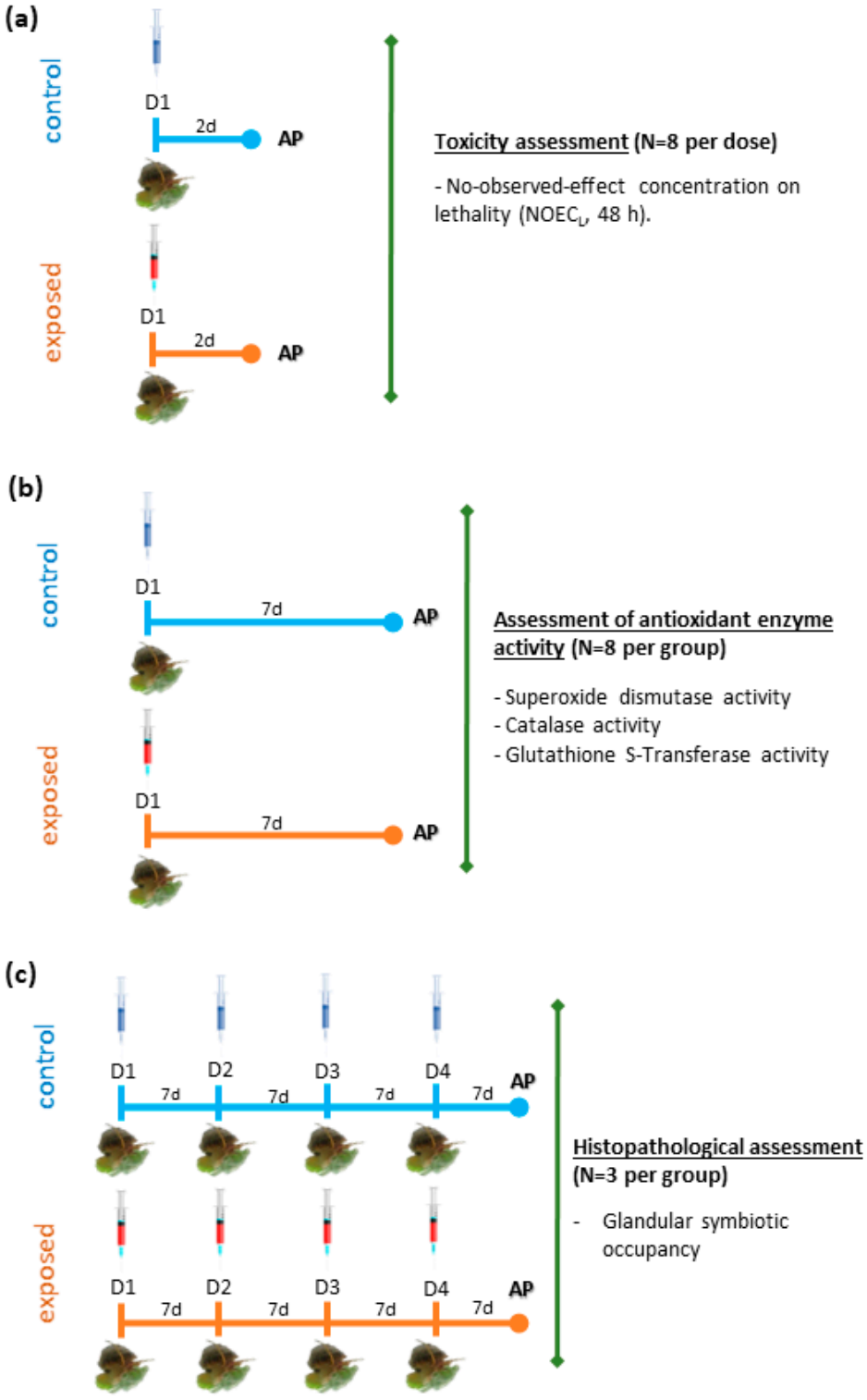


Figure 4. Experimental design to evaluate toxicity, antioxidant enzymes activities, histological alterations in the digestive gland, and symbiotic glandular occupancy in *P. canaliculata* following acute and chronic exposure to the herbicides paraquat (Pq) and fluroxypyr (Fx). The herbicide doses used in (**a**,**b**) were 3.62 µg/g for Pq and 10.42 µg/g for Fx, while in (**c**), the doses were 1.45 µg/g for Pq and 6.94 µg/g for Fx.

4.3.2. Antioxidant Enzymatic Defenses

To assess the effects of acute herbicide exposure on antioxidant enzymatic defenses, five snails per treatment group were injected with a single dose of Pq (3.62 µg/g DM) or

Fx (10.42 µg/g DM), using the $NOEC_L$ concentrations established in Section 4.3.1. The herbicides were dissolved in the vehicle solution, and a control group of five snails received only the isotonic solution. After seven days, all snails were euthanized by immersion in an ice bath (4 °C). Tissue samples from the digestive gland, kidney, and gill were immediately collected and processed for biochemical analysis, as described in Section 4.4. (Figure 4b).

4.3.3. Glandular Symbiotic Occupancy

To evaluate the effects of chronic herbicide exposure, three adult females per group were injected four times, with seven-day intervals between injections. Each snail received an intramuscular injection of Pq (1.45 µg/g DM) or Fx (6.94 µg/g DM), corresponding to their respective tolerated concentrations. At the end of the 28-day exposure period, all snails were euthanized, and tissue samples from the digestive gland were collected for both histological evaluation (see Section 4.5) and morphometric analysis of glandular symbiotic occupancy (see Section 4.6) (Figure 4c).

4.4. Enzymatic Activities

To assess enzymatic activities, 100 mg samples of the digestive gland, whole kidney and gill were homogenized using an UltraTurrax® (IKA Werke, Staufen, Germany) at a 1:4 (*w*/*v*) ratio in a buffer solution containing 20 mM Tris base, 1 mM EDTA, 1 mM dithiothreitol, 0.5 M sucrose, 0.15 M KCl, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.6) [7]. The homogenates were centrifuged at 9000× *g* for 30 min, and the resulting supernatants were used for protein quantification and the determination of SOD, CAT, and GST activities. Total protein concentration was determined using the method described by Lowry et al. [57], with bovine serum albumin as the standard. SOD activity was measured following Misra and Fridovich [58], based on the inhibition of epinephrine auto-oxidation at 480 nm and 30 °C. One SOD unit (U) was defined as the amount of enzyme that inhibits 50% of adrenochrome formation, and activity was expressed as U/mg protein. CAT activity was measured in a 50 mM potassium phosphate buffer (pH 7), following Aebi [59]. The decomposition of 50 mM hydrogen peroxide (H_2O_2) was monitored at 240 nm, and one CAT unit was defined as the amount of enzyme that decomposes 1 µmol of H_2O_2 per minute. CAT activity was expressed as U/mg protein. GST activity was assessed using 50 mM 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in a reaction medium containing 100 mM glutathione (GSH) [60]. The increase in absorbance was measured at 360 nm, and one GST unit was defined as the amount of enzyme required to conjugate 1 µmol of CDNB with GSH per minute. GST activity was expressed as mU/mg protein.

4.5. Light Microscopy

Digestive gland sections (7 µm), obtained as described in Section 4.3.3, were stained using a trichrome stain (Alcian Blue 8GX, Nuclear Fast Red and eosin), in which the nuclei were stained bright red, glycosaminoglycans and C corpuscles were stained deep blue, and cytoplasm was stained from light blue to purple [61]. Micrographs were captured using a Nikon Eclipse 80i microscopy (Nikon Instruments Inc., Tokyo, Japan).

4.6. Glandular Symbiotic Occupancy

Glandular symbiotic occupancy was quantified following the methodology described by Campoy-Diaz et al. [15]. Briefly, digestive gland samples were obtained from three individuals by slicing 1–2 mm thick sections with a razor blade, specifically from the surface near the kidney boundary [48]. The samples were fixed in 4% paraformaldehyde for 24 h and then preserved in 70% ethanol. Tissue dehydration was performed using a graded ethanol series, followed by embedding in Histoplast®. Four to ten sections (7 µm thick) per individual, representing different experimental conditions, were analyzed using

light microscopy. Digital micrographs were captured at 100× magnification with a color video camera mounted on a Nikon Alphaphot-2 YS2 microscope (Nikon Instruments Inc., Tokyo, Japan). Intracellular symbiotic corpuscles (C and K) were identified based on their natural pigmentation and size [47,48] and outlined using Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MA, USA). The occupied area of each corpuscle type (C or K) was measured within individual adenomeres. The mean symbiotic occupancy per individual was calculated and expressed as a percentage of the total epithelial area of each adenomere (set as 100%), excluding the adenomere lumen and corpuscles occasionally seen there. Additionally, the relative proportion of each corpuscle type was determined based on the total symbiotic occupancy.

4.7. Statistical Analysis

Data normality was assessed using the Shapiro–Wilk test, followed by one-way ANOVA and Dunnett's post hoc test to compare control and exposed groups. Analyses were performed using GraphPad Prism® 8.0, with statistical significance set at $p < 0.05$.

5. Conclusions

Our findings show that *P. canaliculata* responds differentially to exposure to the herbicides fluroxypyr and paraquat, exhibiting significant changes in antioxidant enzyme activities and histological alterations in the digestive gland. Tissue-specific enzymatic responses included the decrease in SOD activity in the digestive gland and its increase in the kidney, along with shifts in CAT and GST activity, indicating oxidative stress responses unique to each herbicide. Additionally, histological changes, such as epithelial thickening and vacuolization in Fx-exposed snails, and alterations in glandular symbiotic occupancy in Pq-exposed individuals, suggest a direct impact on the host–symbiont consortium. These different biochemical and morphological effects reinforce the suitability of *P. canaliculata* as a bioindicator species for monitoring aquatic ecosystems affected by agrochemical pollutants. The specific biomarkers identified in our study could be integrated into routine environmental assessments, enabling the early detection of pesticide contamination and supporting more effective management and mitigation strategies in real-world scenarios.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/stresses5020033/s1. Supplementary Table S1: Statistical analysis of enzymatic antioxidant activities in tissues of *Pomacea canaliculata* snails exposed to herbicides.

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