SUBLETHAL EFFECTS OF SPIRODICLOFEN ON NON-TARGET AQUATIC INSECT *Sympetrum meridionale* (ANISOPTERA: LIBELLULIDAE): OXIDATIVE STRESS BUT MINIMAL BIOCHEMICAL ALTERATION

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Abstract. Exposure to agricultural chemical inputs can adversely affect essential biological functions, including growth, reproduction, and various morphological and physiological traits in living organisms. In this study we aim to evaluates the effects of sublethal exposure to the insecticide spirodiclofen on the survival patterns and three key biochemical parameters - malondialdehyde (MDA) levels, total lipid content, and total protein content - of an aquatic insect species, the Southern darter *Sympetrum meridionale* (Anisoptera: Libellulidae) stemming from an agroecosystem located of northeastern Algeria. Larval stages of the species were subjected to a range of spirodiclofen concentrations (control, 0.6, 1.5, and 12 µg /L) over a two-week bioassay period to assess dose-dependent physiological and biochemical responses. Because 50% mortality was not observed at the tested concentrations, the LD_{50} was obtained through model-based extrapolation and estimated at 45.34 µg/mL ($CI_{95\%}$: 10.41, 202.72). According to the Cox proportional hazards model larvae exposed to the highest dose of the insecticide experienced a mortality rate approximately three times greater than that observed in the control groups. The one-way ANOVA indicates that exposure to spirodiclofen did not significantly affect the protein (df = 3, F = 1.40, p = 0.27) and lipid (df = 3, F = 0.57, p = 0.64) content in the larvae across the treatment groups. In contrast, malondialdehyde (MDA) content showed a significant difference (df = 3, F = 12.45, p < 0.001). These findings suggest that a single pulse exposure to spirodiclofen induced oxidative stress, but did not pose a critical risk to *S. meridionale* larvae, where by the end of the experiment (16 days) survival declined to about 50% at 0.6 and 1.5 µg/mL and 30% at 12 µg/mL. This underscores the importance of evaluating both acute and chronic toxicity in ecological risk assessments, especially for non-target aquatic organisms in environments subjected to recurrent pesticide applications.

Keywords: dragonfly; insecticide; physiology; toxicity; wetland.

INTRODUCTION

When a pesticide is applied, it does not all reach its intended target. Instead, it undergoes a range of environmental processes [64]. A portion may evaporate into the atmosphere, while some is absorbed by plants or animals that come into contact with it. Only a fraction typically reaches and impacts the target pest species. Additionally, pesticides can settle into the soil, where they may degrade over time, or leach into groundwater and run off into nearby surface water [11]. Wetlands, especially those embedded in agricultural landscapes such as freshwater ponds, are among the most vulnerable non-target areas, consistently facing a pressure from various contaminant inputs including pesticides [12]. It is true that these ecosystems have the capacity to remediate pesticides, but as the contaminant load continues to increase and climatic conditions become harsher (droughts), these ecosystems lose their self-purifying ability, and many aquatic organisms will be threatened, leading to global biodiversity loss [65, 69]. Environmentalists, scientists, and agricultural professionals are well aware of the potential risks that pesticide residues pose to non-target areas [5, 42, 52]. However, there remains a continual need for updated information and environmental monitoring on the effects of these agrochemicals on various organisms, especially in regions facing intensive agricultural activity and unpredictable climate variability [45].

The use of toxicity data - both acute and sublethal - is essential for ecotoxicological assessments of chemicals and is recommended to strengthen current toxicity databases for various aquatic organisms [46]. Due to their well-documented ecological characteristics, odonates (dragonflies and damselflies) are considered suitable test species in ecotoxicological studies [29]. Agrochemicals are highly effective in the field, causing rapid insect mortality. However, over time, they degrade into sublethal concentrations in the environment [54]. Since odonates are non-target species and typically inhabit areas adjacent to pesticide application zones, they are more likely to experience sublethal effects than acute toxicity. Bioassays involving these species could enhance our understanding of the consequences of sublethal insecticide exposure on multiple life-history traits in non-target organisms.

Spirodiclofen, a tetronic acid derivative, has recently been commercialized as an acaricide highly effective against all relevant phytophagous mite species [39]. Environmental monitoring has revealed the presence of spirodiclofen in river sediments at concentrations of 0.881 mg/g [70], although its solubility in freshwater is limited to 50 µg/L at pH 4 [31]. It is a broad-spectrum acaricide that differs from other insecticide in its biochemical mode of action, functioning as a lipid biosynthesis inhibitor that disrupts vital metabolic processes in insects [19, 22]. Exposure to sublethal concentrations of this acaricide such as any chemicals can trigger energy-demanding detoxification processes such as oxidation and hydrolysis, which may lead to oxidative stress and cellular damage. As a result, essential biological functions - including growth, reproduction, and various

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morphological and physiological traits can be adversely affected [42].

The present study aims to evaluate the sublethal effects of the commercial formulation spirodiclofen (ENVIDOR® 240 SC) on the larval survival and three biochemical parameters malondialdehyde MDA, lipid, and protein content, in the non-target species *Sympetrum meridionale* (Anisoptera: Libellulidae) from an agroecosystem in northeastern Algeria. *S. meridionale* is a widespread and abundant dragonfly of the Mediterranean regions. It lives near stagnant and shallow waters with abundant emergent vegetation. For reproduction *S. meridionale* needs shallow water bodies with low water-level for oviposition and flooding during the subsequent months until emergence in the following year [27, 51]. This aquatic insect species, along with other congeneric Odonata taxa, plays a key ecological role as an intermediate predator in both aquatic and terrestrial ecosystems [66]. They help regulate populations of smaller invertebrates, including pest species, and serve as relevant bioindicators of environmental changes, such as pesticide contamination [43, 68]. Understanding how these species respond to environmental changes is essential for addressing conservation risks in a timely manner [58]. Although spirodiclofen is no longer authorized within the European Union, it remains in use in several third countries, including ours [21]. Importantly, data on the biochemical responses of Odonata and other non-target organisms to spirodiclofen are extremely limited. Conducting such a study is particularly significant in the southern Mediterranean basin, where comparable research is scarce and climate conditions are increasingly unpredictable

MATERIAL ADN METHOD

Insect collection and rearing

Eggs of *S. meridionale* were obtained from copulating or ovipositing females captured using aerial insect nets [53]. To collect eggs, each female's abdomen was gently dipped into a tube containing water [7]. The females were collected from a freshwater pond in an agricultural region of northeastern Algeria (36º22'05'' N; 7º24'47'' E). Eggs were submerged in spring water in small container (9 × 15 × 22 cm^3) maintained at 20 ± 1 ºC, with water occasionally added to maintain a minimum depth of 3 cm [37]. Freshly hatched larvae were reared individually in 200 ml cups with a constant photoperiod of (14:10:L:D). Larvae were daily fed with *Artemia nauplii* for the entire duration of the experiment. When larvae molted into the final instar, they were randomly allocated in groups and used for the toxicity experimental trials.

Experimental design and treatments

The bioassay consisted of a single-pulse exposure to trade formulation of spirodiclofen (ENVIDOR® 240 SC, Bayer Algeria SPA) at a series of four concentrations (0 - 0.6 - 1.5 - 12) µg/L, to ensure non-lethal effects and to cover the expected effect range. Each treatment comprised three (3) replicates; each replicate consisted of 10 larvae maintained individually in 200 ml cups filled with spring water (30 larvae per treatment).

Control larvae received 1 µL of solvent alone (to avoid solvent effects, the volume of acetone introduced into test cups was kept ≤0.01% , acetone/water: 50/50). However the concentration of acetone used in such studies is not expected to be harmful [48]. The insecticide was diluted in acetone and 1 mL of working solution was placed in each cup and water was not changed over the experimental period. At the end of the experiment each larva was individually stored in a -80° C freezer for biochemical analysis.

Biochemical analysis

MDA (malondialdehyde) levels in samples were measured using the thiobarbituric acid reaction method of Draper and Hadley [18]. Quantification of thiobarbituric acid reactive substances was determined at 532 nm by comparing the absorption to the standard curve of MDA equivalents. Values of MDA were expressed as nmol/mg protein [26].

Lipid and protein were extracted following the procedure of Shibko *et al.* [57] and quantified as described in Soltani-Mazouni, *et al.* [59]. Each larva was weighed and extracted in 1 mL of trichloracetic acid (20%). The total of proteins based on the Bradford method [8] using bovine serum albumin as standard (BSA, Sigma), and of lipids based on the vanillin method, were also quantified [50].

Statistics

During our bioassay 50% mortality was not observed, thus the sublethal concentrations (Lethal dose LD10%, 20%, 30% and 50%) estimation required extrapolation beyond the range of observed mortalities; therefore, its accuracy depends on the assumption that the logistic model adequately characterizes the dose–response relationship outside the tested concentration range. This limitation introduces additional uncertainty, and the obtained value should be interpreted as a model-derived prediction rather than an experimentally verified threshold using a logistic procedure. These concentrations were determined following exposure of *S. meridionale* larvae to a single doses of the test substance, spirodiclofen, within 24 hours [20].

Mortality rate was compared for each larval group (30 larvae per treatment) tested using a standard survival analysis, with Kaplan–Meier estimates. We utilized the survival analysis implemented in the survival packages [24] for R Commander [23] to identify significant effects of pesticide concentration on survival rates, we used the Cox proportional hazards model. In the models, insect ID was entered as a random effect using the "frailty" function [60] whereas insecticide concentration with four levels (0, 0.6, 1.5, 12 µg/L) was inserted as the fixed component of the models. Before interpreting the Cox model, we verified

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the proportional hazards assumption using Schoenfeld residual diagnostics (“*cox.zph”* function in R). No significant deviations from proportional hazards were detected for concentration covariate and the global test (all p-values > 0.05). We also inspected deviance residuals to check for influential observations

To identify significant effects of pesticide concentration on the three biochemical parameters (Protein, MDA and lipid) we used one way analyse of variance ANOVA. Insecticide concentration with four levels (0, 0.6, 1.5, 12 µg/L) was inserted as the fixed factor for all analysis. Prior to analysis, data were checked for normality (Shapiro–Wilk test) and homogeneity of variance (Levene’s test). Lipid and MDA data met normality assumptions ($p > 0.05$), while Protein data showed a slight deviation from normality ($p = 0.03$), but inspection of residual plots indicated no substantial departures from normal distribution, and variances were homogeneous among groups. Given the robustness of ANOVA to minor normality violations, we applied one-way ANOVA to all three biochemical parameters. ANOVA was followed by Tukey’s HSD post-hoc test to identify pairwise differences among concentrations.

RESULTS

The sublethal concentrations for *S. meridionale* were LD10 (7.01 µg/mL, $CI_{95\%}$: 3.33, 14.77), LD20 (13.97 µg/mL, $CI_{95\%}$: 5.98, 32.61) and LD30 (22.08 µg/mL, $CI_{95\%}$: 7.64, 63.78). Estimated LD 50 for the species was 45.34 µg/mL ($CI_{95\%}$: 10.41, 202.72) (Figure 1).

The effects of different concentrations of spirodiclofen on the survival of larvae that were treated with spirodiclofen are depicted in figure 2. Survival

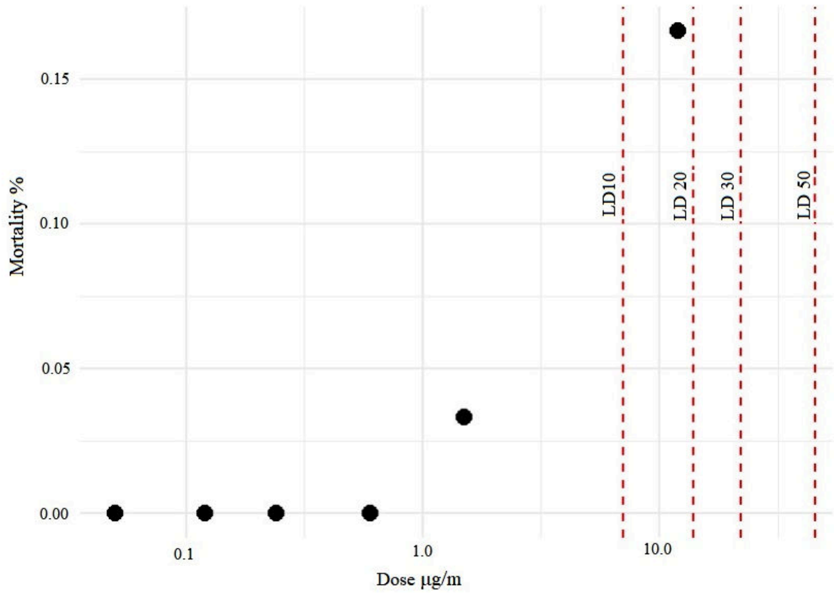

Figure 1. Effect of spirodiclofen on mortality of *S. meridiona*l larva: Determination of lethal doses LD 10, LD 20, LD 30 and LD50

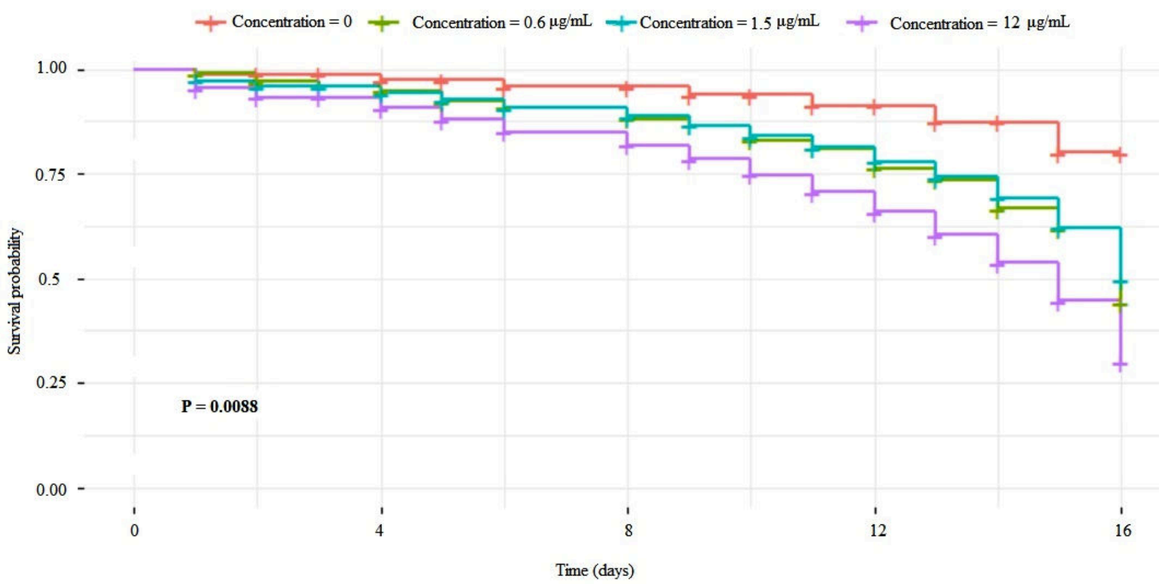

Figure 2. Effect of different concentrations of spirodiclofen on larval mortality of *Sympetrum meridionale*.

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Table 1. Mortality of *Sympetrum meridionale* larvae treated with different concentrations of the spirodiclofen insecticide

Dose	HR	SE	Chi square	DF	P_value
Spirodiclofen (0.6 m/L)	2.88	0.43	5.37	1	0.02
Spirodiclofen (1.5 m/L)	2.59	0.45	4.37	1	0.03
Spirodiclofen (12 mg/L)	4.39	0.45	10.46	1	0.001
Insect ID			1.96	0.7	0.9

HR: hazard ratio, *SE* standard error, *df* degrees of freedom
Concordance= 0.735 (SE = 0.039)
Likelihood ratio test= 12.31 on 3 df, p=0.008
N = 120 larvae, evaluation period = 16 days.

was significantly lower in the groups treated with the insecticide with respect to the untreated groups. According to the Cox proportional hazards model, all applied doses of spirodiclofen had a significant effect on mortality risk, indicating a dose-dependent increase in hazard ratios (Table 1). Over the course of two weeks, larvae exposed to the highest dose of the insecticide experienced a mortality rate approximately three times greater than that observed in the control groups (Figure 2). Survival curves further illustrate this pattern: control larvae maintained the highest survival with approximately 80% remaining alive by day 16, while survival declined to about 50% at 0.6 and 1.5 µg/mL µg/mL. The steepest decline occurred at 12 µg/mL, where only around 30% of larvae survived by the end of the experiment (Figure 2). This marked increase in mortality indicates a dose-dependent response, with the highest insecticide concentration substantially reducing larval survival. However, no total mortality was observed by the end of the experiment.

The analysis revealed that protein and lipid levels in *S. meridionalis* larvae were similar between the control groups and those treated with spirodiclofen (Figure 3). The one-way ANOVA indicates that exposure to spirodiclofen did not significantly affect the protein (df = 3, F = 1.40, p = 0.27) and lipid (df = 3, F = 0.57, p = 0.64) content in the larvae across the treatment groups. In contrast, there was a significant increase in MDA levels with increasing pesticide concentration (F = 12.54, P < 0.001). Tukey HSD tests showed that MDA levels were significantly higher at 1.5 m/L (P = 0.0025) and 12 mg/L (P < 0.001) compared to the control, while differences between 0.6 m/L and control, and between other pairs, were not statistically significant (Figure 3).

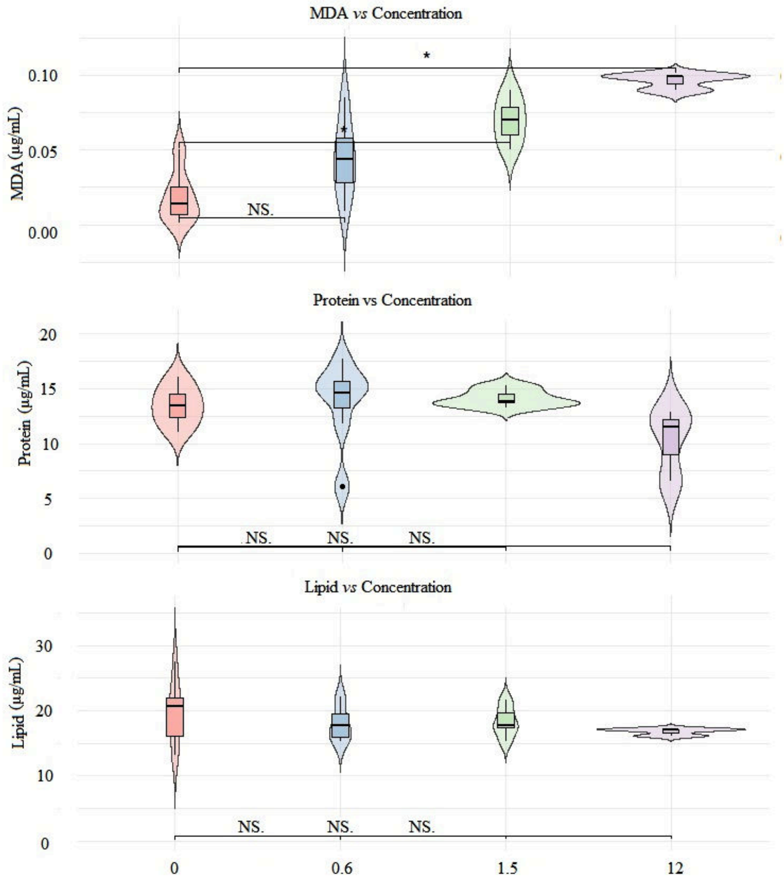

Figure 3. Effects of pesticide concentration on biochemical parameters (Protein, MDA and lipid µg/ml) in *S. meridionale* larvae. (*) indicate significance, NS not signeficat (ANOVA, Tukey's HSD post-hoc)

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DISCUSSION

In this study, we investigated the effects of exposure to different concentration of spirodiclofen on the survival rate and three vital biochemical parameters (proteins, lipids and MDA contents) of a non-target insect species, the Southern darter, *Sympetrum meridionale*. We found that insecticide exposure elicited significant negative effects on the survival rate of *S. meridionale* larvae, showing a clear dose-dependent response, with the highest concentration substantially reducing larval survival. It is widely recognized that all types of insecticides can adversely impact insect growth and development [56]. Our results align with numerous studies that document the harmful effects of pesticides on non-target insects such as honey bees [30, 41], damselfly [60, 66] and dragonfly [33]. A similar outcome has been also observed for spirodiclofen on other invertebrates such as earthworms [13, 38] and arthropods such as wolf spider [36]. However, the intensity of these effects can vary depending on the insect species and the specific insecticide used. The LD_{50} (45.34 µg/mL) obtained in this study is much higher i.e. lower toxicity compared with previous researches on the non-target effects of spirodiclofen on various natural enemies in which this insecticide has been classified as harmful, for instance the predatory thrips *Scolothrips longicornis* (LD50 = 9.86 µg/mL) [26] and adults of *Amblyseius swirskii* (11.96 µg/mL) [4]. Also, The LD_{50} value of 45.34 µg/mL obtained in this study exceeds the Predicted Environmental Concentration in Surface Water (PECSW) of 14 µg/L for a standard application scenario with 3 m buffer zones [63]. This finding indicates that the concentrations likely to occur in surface water under standard application practices are below the acute toxicity threshold for *S. meridionale* larvae. More precisiley, the measured acute LD_{50} of spirodiclofen for *S. meridionale* (45.34 µg/mL, equivalent to 45,340 µg/L or 45,340,000 ng/L) is several orders of magnitude higher than both the pesticide residues detected in fruits (strawberry: 2 ppm; tomato: 0.5 mg/kg; citrus: <0.2 mg/kg) and typical environmental water concentrations (1–1,000 ng/L) [1, 2, 61]. The fold differences between LD_{50} and fruit residues range from ~23-fold for strawberry to >226-fold for citrus, while water concentrations are roughly 45,000–45,000,000-fold lower than the LD_{50}. This comparison assumes complete bioavailability and water equivalence; real environmental water concentrations are often lower due to dilution, degradation, and metabolism. This indicate that even in the most extreme environmental scenarios, waterborne spirodiclofen concentrations are far below the lab LD_{50}, although chronic or sublethal effects at low environmental concentrations cannot be excluded. The obtained LD_{50} was not directly observed during the bioassay, as 50% mortality did not occur within the experimental conditions. However, it was extrapolated using logistic regression, a common method for estimating the lethal dose when a complete dose-response curve cannot be observed in a limited experiment. This extrapolation should be interpreted cautiously, as it assumes that the model can accurately predict mortality at doses beyond those tested. Despite this limitation, this finding can contribute to a better understanding of the environmental risks posed by this pesticide, particularly to a rarely investigated non-target species like *S. meridionale* larvae.

According to the Cox proportional hazards model our results indicate also that the acaricide spirodiclofen has relatively low toxicity for *S. meridionale* larvae compared to other insecticides applied to similar taxa. For instance, a single pulse application of cypermethrin and diflubenzuron (separately) at 0.015 mg/L resulted in nearly complete mortality of *Ischnura elegans* larvae within the first eight days [60]. In contrast, our larvae exposed to a comparable concentration of spirodiclofen (0.012 mg/L) reached a 50% mortality rate only after approximately two weeks. Lower toxicity of the studied acaricide was also reported against larval development of other predators such as Ladybird beetle [3] and *Orius niger Wolf* [44]. This suggests that spirodiclofen, while still harmful, may have a less immediate impact on non-target species compared to other insecticides. Indeed, in various studies spirodiclofen being classified “slightly hurmful” [10, 25, 67]. The lack of total mortality observed by the end of the experiment and higher survival rates at the three first days (more than 75%) suggests that, while high doses of the insecticide are detrimental, *S. meridionale* larvae demonstrate a notable level of tolerance and may be capable of adapting to otherwise unfavorable conditions. This resilience is not surprising, as numerous studies have highlighted the adaptive plasticity of odonates in response to habitat changes [14, 48, 49]. Additionally, odonates, including *S. meridionale*, are known for their ability to adjust to environmental stressors, which may explain their tolerance to sublethal levels of insecticides observed in this study [28, 35, 47, 49].

Numerous studies have been conducted to compare toxicity and biochemistry mechanisms in target and non-target insects [17]. The use of pesticide could significantly affect levels of proteins, lipids, and carbohydrates in insects [3]. In this study, and contrary to expectations, spirodiclofen exposure did not affect lipid and protein levels in the treated *S. meridionale* larvae. The lack of change in protein levels in treated larvae is logical, as this member of spirocyclic tetronic acaricide family primarily targets lipid biosynthesis by directly inhibiting acetyl-CoA carboxylase, the initial enzyme on the path of fatty acid biosynthesis [6, 9, 16]. This enzyme is critical for lipid metabolism rather than protein synthesis, so the pesticide's mechanism of action would likely spare protein levels while disrupting lipid pathways. While spirodiclofen targets lipid metabolism, our results showed stable lipid levels in the treated larvae. It appears that a single pulse application of this insecticide was not potent enough to

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induce measurable disruptions in lipid metabolism within the study period. Importantly, the concentrations and the exposure duration used in this experiment reflect realistic scenarios commonly found in freshwater ecosystems, where spirodiclofen often persist at low concentrations and for limited periods [15]. This unexpected stability may be a transient effect, potentially dependent on the duration of exposure. Longer or repeated exposure might disrupt lipid levels more significantly as the inhibition of lipid biosynthesis progresses over time. Thus, the single pulse application of this insecticide was not high enough to induce lipid metabolism disruptions. The absence of detectable effects of spirodiclofen on total proteins and lipids may also be related to the fact that aquatic insect larvae are known for their strong metabolic plasticity and for possessing highly efficient detoxification systems. In particular, mixed-function oxidases such as cytochrome P450 monooxygenases (CYPs) and carboxylesterases (CaEs) can rapidly biotransform and hydrolyze xenobiotics, thereby preventing pesticides from accumulating to levels that would alter basic biochemical constituents [34]. These detoxification pathways not only protect the organism by preventing the accumulation of toxic compounds, but in some species they also enable significant biotransformation of pesticides. As a result, certain aquatic and terrestrial invertebrates can serve as natural bio-remediators, contributing to the degradation and removal of pesticides from polluted soils and water bodies [55]. On the other side, malondialdehyde (MDA) levels showed a significant increase in response to pesticide exposure. This selective effect suggests that, although basic macromolecular reserves remained stable, the pesticide induced oxidative stress, as evidenced by elevated MDA, a marker of lipid peroxidation. This increase in MDA indicates cellular oxidative damage, likely due to the stress of detoxification processes [32].Although total lipids and proteins were not significantly altered, the observed increase in MDA clearly indicates enhanced lipid peroxidation and oxidative stress. This suggests a physiological trade-off, where antioxidant defenses and detoxification pathways may temporarily protect macromolecules but still allow measurable oxidative damage. Such sublethal oxidative stress may carry long-term implications for energy allocation (decreased of energy reserve), growth, DNA damage, and overall fitness, even in the absence of immediate depletion of major biochemical constituents [40, 62].

Spirodiclofen exhibits low sublethal toxicity to *S. meridionale* larvae, as evidenced by its relatively high LD_{50} value and the absence of a significant increase in mortality during the first week of exposure. Furthermore, the insecticide does not appear to disrupt lipid and protein biosynthesis, which is consistent with its mode of action targeting acetyl-CoA a key enzyme in lipid metabolism. The most notable biochemical effect was the alteration of malondialdehyde (MDA) levels, suggesting oxidative stress likely triggered by detoxification processes. Such oxidative damage, while not immediately reflected in major biochemical pools, may impose long-term physiological costs under chronic or repeated exposure. These findings highlight the importance of considering subtle biochemical disturbances, not only overt lethality, when evaluating pesticide impacts on non-target aquatic organisms. Incorporating indicators of oxidative stress and sublethal effects into ecological risk assessments would strengthen pesticide regulation and provide a more realistic understanding of long-term environmental hazards. In this study Spirodiclofen appears less disruptive to physiological homeostasis and survival compared to organophosphates or pyrethroids previously tested on Odonata larvae, spirodiclofen thus appears less disruptive to physiological homeostasis and survival, reinforcing thereby its profile as a relatively selective acaricide with reduced non-target impact under limited exposure. These findings suggest that a single pulse exposure to spirodiclofen under the tested conditions does not pose a critical risk to *S. meridionale* larvae. However, it is essential to consider that longer or repeated exposures may amplify toxic effects, potentially resulting in greater harm over time. This underscores the importance of evaluating both acute and chronic toxicity in ecological risk assessments, especially for non-target aquatic organisms in environments subjected to recurrent pesticide applications.

Conflict of interest. There is no actual or potential conflict of interest in relation to this article

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