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Efficacy of Phosphine on Different Life Stages of *Alphitobius diaperinus* and *Tenebrio molitor* (Coleoptera: Tenebrionidae)

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Abstract: The efficacy of phosphine has been established for numerous major stored product insects. However, data related to the evaluation of the effect of phosphine on *Tenebrio molitor* L and *Alphitobius diaperinus* Panzer are limited. The present study aims to evaluate the susceptibility of these species to phosphine by using the following evaluation protocols: (a) all life stages were exposed for 3 days to different concentrations of phosphine, (b) adults were exposed to 3000 ppm until all exposed individuals were immobilized, using the Phosphine Tolerance Test (PTT, Detia Degesch GmbH, Germany), and (c) adults were exposed to 3000 ppm of phosphine for 90 min by again using the PTT protocol. For all series of bioassays, delayed mortality was recorded 7 and 14 d post-exposure. According to our results, 100 ppm for three days was sufficient to kill all life stages, including the eggs, for both species. *Alphitobius diaperinus* adults were found to be more tolerant than those *T. molitor*, as noticeable survival was observed, even after 90 min of exposure to 3000 ppm. Our study provides some initial data for the efficacy of short and long exposures of *A. diaperinus* and *T. molitor* to phosphine.

Keywords: phosphine; mealworms; tolerance

Citation: Gourgouta, M.; Athanassiou, C.G. Efficacy of Phosphine on Different Life Stages of *Alphitobius diaperinus* and *Tenebrio molitor* (Coleoptera: Tenebrionidae). *Sustainability* **2023**, *15*, 2131. https://doi.org/10.3390/su15032131

Academic Editor: Sean Clark

Received: 11 November 2022
Revised: 4 January 2023
Accepted: 14 January 2023
Published: 23 January 2023

1. Introduction

Phosphine gas (PH3) is the key fumigant for the control of insects infesting durable agricultural commodities during storage and processing [1–3]. The importance of this active ingredient has increased over the last decades due to its numerous advantages over the use of other methods [3]. The most important advantages of this insecticide are its low cost and ease of application, its characterization as a residue-free treatment, and its high efficacy against a wide range of major pests infesting stored products [3,4].

As in the case of the vast majority of insecticides that are currently in use, there are considerable variations in the efficacy of phosphine among different target insect species and different life stages of the same species [3]. In this context, a natural tolerance of some species has been observed that is not related to the development of resistance due to previous exposure to phosphine [5,6]. For instance, Gautam et al. [6] reported that the LC99 values of the eggs of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), and the Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), were 51.5 and 84.4, respectively. Moreover, Athanassiou et al. [7] found differences in the susceptibility to phosphine of different life stages of the larger cabinet beetle, *Trogoderma inclusum* LeConte, and the hide beetle, *Dermestes maculatus* DeGeer (Coleoptera: Dermestidae). The majority of the data available so far underline that, for most of the species tested, eggs should be considered as the life stage most tolerant to phosphine [3]. Hence, when conducting bioassays to estimate the susceptibility to phosphine, eggs should be included, as this is the life stage that is most likely to survive and cause a rapid population rebound that will continue the infestation in a very short period. The same holds in the case of bioassays that are carried out in the field or in

semi-field conditions, given that using adults or larvae alone might provide the impression that the application is effective [8,9]. Collecting eggs is not always possible, as it is a demanding and laborious procedure, but these can be replaced with vials containing adults and commodities that have been left for a certain period to oviposit, which is an approach that can be easily utilized at the industrial level [9–12].

Several protocols have been developed for the detection of resistance of stored product insects to phosphine and for the quantification of the efficacy of this gas in "real world" applications [3,13]. The most commonly used protocol is the Food and Agriculture Organization (FAO) test, which was initially proposed by Champ and Dyte [14]. In this protocol, the immediate effect of phosphine is evaluated after a 3-day exposure interval to 30 ppm of phosphine, and subsequent mortality is observed 7 and 14 days later [15–17]. Variations of this protocol are used as the "Dose Response" protocols, which are based on exposing individuals for 3 days at different concentrations [6,18,19]. Other protocols, such as the Phosphine Tolerance Test (PTT, Detia Degesch GmbH, Germany), rely on the "speed to immobilization", providing quick data on the reduced susceptibility to phosphine [3,13,20]. Athanassiou et al. [20] evaluated the susceptibility to phosphine of different populations of thirteen species, obtained from different laboratories in different countries, using immobilization as a quick diagnostic indicator for resistance and providing information for a potential quick indicator of resistance development.

Although the aforementioned protocols have been used and reported for a wide range of species, there are no studies to indicate their utilization for the evaluation of the efficacy of phosphine against the yellow mealworm, *Tenebrio molitor* L., and the lesser mealworm, *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae). These species have received great attention in recent years, as they are among the most promising species for mass production and utilization as food and feed [21–24]. Both species have been detected in a wide range of stored products [23], and *A. diaperinus* is a potential carrier of various avian pathogens that cause dangerous diseases to poultry and humans [24–26].

Taking into consideration the importance of these two species, and the fact that the data available so far for the efficacy of phosphine for their control are scarce, the present study aims to evaluate the immediate and delayed efficacy of phosphine at different life stages of *A. diaperinus* and *T. molitor* using different protocols of short and long exposures.

2. Materials and Methods

2.1. Insects

Individuals of both species were obtained from populations maintained at the Laboratory of Entomology and Agricultural Zoology (LEAZ) at 26 ± 1 °C, 55% relative humidity (r.h.), and continuous darkness. Rearing was carried out in a mixture of one-fifth dry instant yeast (Angel Yeast Co. Ltd., Yichang, China) and four-fifths wheat bran and supplemented with fresh potato slices twice a week, which were used as a moisture source [27].

Adults, pupae, and large larvae of *T. molitor* were separated from the rearing substrate using a 2 mm sieve, and small larvae were separated using an 850 µm sieve. Similarly, regarding *A. diaperinus*, adults and pupae were separated from the rearing substrate using a 2 µm sieve. For the separation of large and small larvae, we used a 1 mm and 650 µm sieve, respectively. After the separation of the rearing substrate, individuals in all life stages were collected with a fine paint brush (lineo, No.1, Mesko-Pinsel GmbH, Wieseth, Germany). For both of the species examined, eggs were obtained by placing approx. 100 g of adults in white wheat flour to oviposit for a week. Thereafter, adults were removed, and eggs were collected from the oviposition substrate using a 250 µm sieve.

2.2. Bioassay I

All life stages were tested in this bioassay, i.e., adults, pupae, large larvae, small larvae, and eggs. Ten individuals from each of the different life stages were placed in plastic cylindrical vials (2.5 cm in diameter, 9 cm in height, different series of vials for each life stage). Subsequently, all vials were transferred in 1 L jars which were used as experimental

chambers. The gas production was carried out as proposed by Steuerwald et al. [28], and then injected through a gas-tight rubber septum at the lid of the jar using a volumetric syringe to achieve the desired concentration, which was defined as either 50 or 100 ppm. Additional jars, with insects, that contained only air were used as controls. All jars were placed in incubators set at 28 °C and 55% r.h. Three days later, the vials were opened, and the mobile stages, i.e., adults, small larvae, and large larvae, were recorded as active (showing visible movement) or immobilized (with no visible movement). All individuals were removed from the vials and placed in Petri dishes with small quantities of wheat bran, set at 28 °C and 55% r.h. The Petri dishes were supplemented with carrot slices as a moisture source 3 times a week. Seven days later, all adults and larvae (both small and large) were observed for mortality. Eggs were observed for hatching, while pupae were observed for adult emergence. The same procedure was also repeated 14 days after the exposure. There were 2 replicates with 3 subreplicates (6 jars in total) for this test, with new phosphine production each time.

2.3. Bioassay II

In this series of bioassays, adults of both species were tested using the PPT protocol. Phosphine production was conducted as described by Agrafioti et al. [13]. Five adults each were introduced into 100 ml syringes, and all syringes were then filled with 3000 ppm of phosphine. The individuals were recorded visually every 2 min until all exposed individuals were immobilized. Syringes with adults, containing only air, were used as controls. When 100% of the exposed individuals were immobilized, they were removed from the syringe and transferred to Petri dishes in the open air with a small amount of food, along with carrot slices, as above. Mortality was recorded 7 and 14 days later. There were 3 replicates with 3 subreplicates for each species (9 syringes for each combination).

2.4. Bioassay III

In this series of bioassays, adults of both species were exposed to 3000 ppm for 90 min following the procedure described in Bioassay II. After the termination of this interval, all individuals were transferred to Petri dishes in which the immediate response was observed and classified either as active or as immobilized, and delayed effects were recorded 7 and 14 days later.

2.5. Statistical Analysis

For Bioassay I, as 100% of insects from all stages examined were immobilized after 3 days of exposure, no statistical analysis of the results for the immediate effect was performed. Regarding the delayed effect (the percentage of mortality of the eggs), the data were not normally distributed, nor could a transformation be found that allowed the presumption of normality. Non-parametric Wilcoxon tests were used to assess rank differences among the treatments with respect to each variable. Pairwise comparisons among the treatments were performed using a Wilcoxon 2-sample test in order to compare egg mortality at 50 and 100 ppm separately for both post-exposure intervals (7 and 14 days post-exposure) and species. The percentage of mortality for all the life stages tested was 100% for both post-exposure periods. In contrast, 0% mortality was noted for the control insects of all life stages for all intervals. Therefore, no further analysis was performed for this bioassay. Regarding Bioassay II, data were analyzed using probit analysis to estimate the lethal time for killing 50, 95, and 99% of the exposed individuals, i.e., LT50, LT95, and LT99 for both species tested. Finally, for Bioassay III 90, all data were submitted to *t*-tests separately for each interval to compare adult mortality of the species tested. For all the series of bioassays, regarding delayed efficacy, all data were submitted to *t*-tests for both post-exposure intervals (7 and 14 days post-exposure time).

3. Results

3.1. Bioassay I

All adults and larvae were immobilized after 3 days of exposure at 50 and 100 ppm (Figure 1). In addition, 100% mortality was observed for both species 7 and 14 days later for both adults and larvae. In contrast, some survival was recorded when eggs were exposed to 50 ppm of phosphine, since 95% of the eggs of *A. diaperinus* were considered dead, as they did not result in larval hatching in the 7-day post-exposure period, a percentage that was only slightly increased 7 days later (Figure 1). Regarding the *T. molitor* eggs, the mortality levels were 91.6 and 88.8% after 7 and 14 days, respectively (Figure 2). Eggs that had been exposed at 100 ppm of phosphine did not result in larval hatching for either of the species tested (Figures 1 and 2).

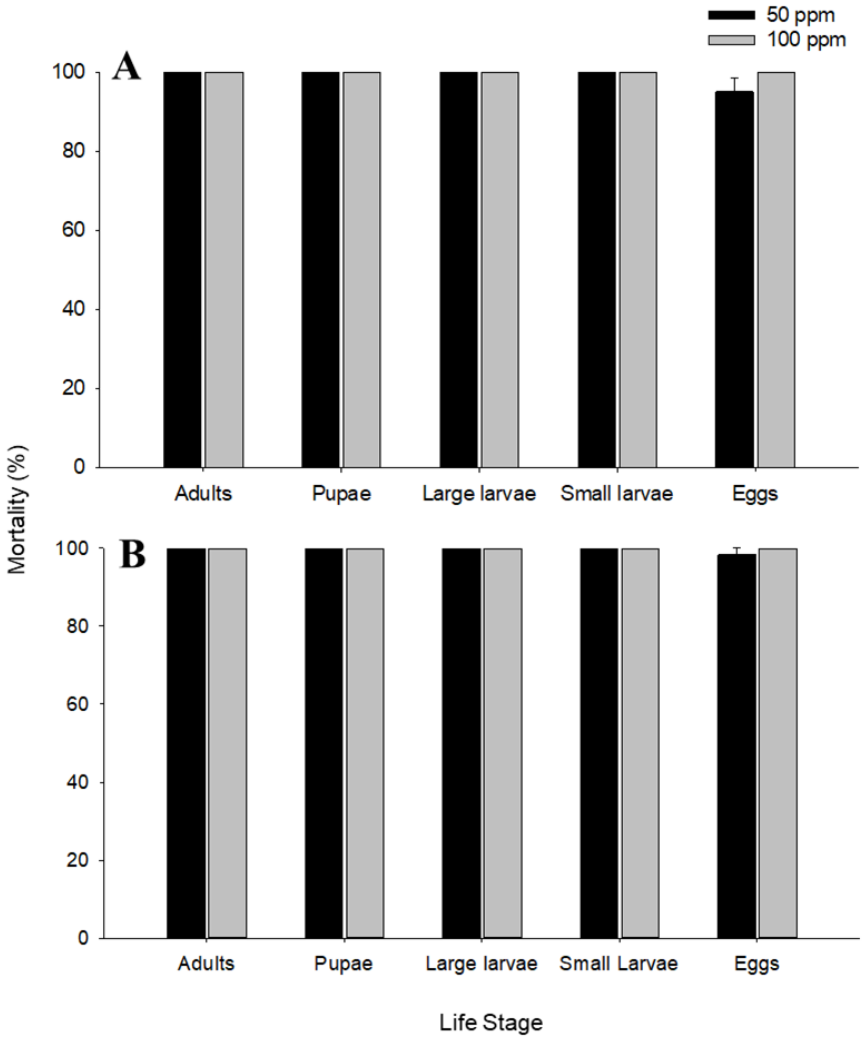

Figure 1. Mean number (% ± SE) of dead adults, pupae, large larvae, small larvae and eggs of *A. diaperinus* 7 (**A**) and 14 days (**B**) after the termination of the 3-day exposure to 50 and 100 ppm of phosphine (P7 = 0.17, Z7 = −1.34; P14 = 0.40, Z14 = −0.83).

3.2. Bioassay II

Probit analysis fit the data adequately for both species (Table 1). LT99 was determined as 5.54 and 4.34, whereas LT50 was 2.44 and 1.78 for *A. diaperinus* and *T. molitor* adults, respectively. However, regarding the 7-day post-exposure period, adult mortality of *T. molitor* was approx. 60%, i.e., three times the mortality rate of *A. diaperinus*. Furthermore, adult mortality of *A. diaperinus* was doubled at the 14-day post-exposure period, reaching 40%, but remained much lower than that of *T. molitor* adults, which reached 93.3% (Table 2).

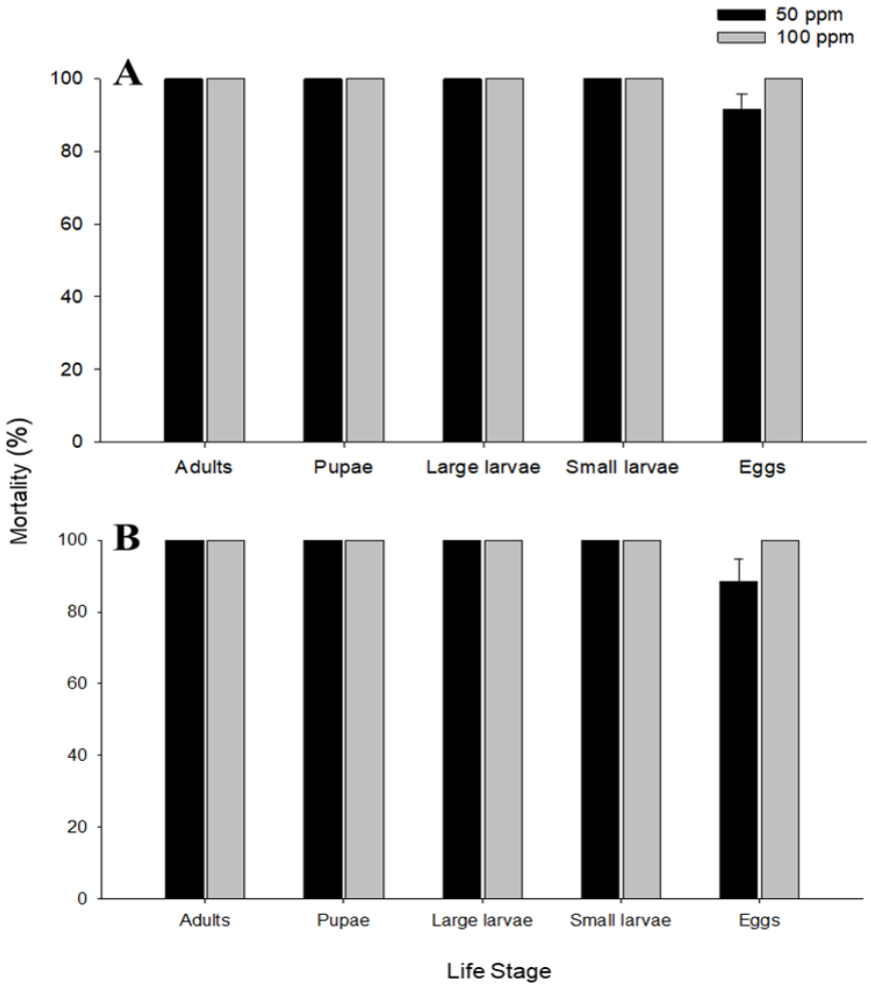

Figure 2. Mean number (% ± SE) of dead adults, pupae, large larvae, small larvae, and eggs of *T. molitor* 7 (**A**) and 14 days (**B**) after the termination of the exposure to 0 ppm (control), 50 ppm, 100 ppm for 3 days (P_7 = 0.07, Z_7 = −1.79; P_{14} = 0.7, Z_{14} = −1.78).

Table 1. Probit analysis for LT_{50}, LT_{95}, LT_{99} (confidence intervals) of adults of *A. diaperinus* and *T. molitor* after exposure to 3000 ppm concentration for the insect population tested, expressed as minutes to immobilization, using the PPT protocol.

Species	LT_{50}	LT_{95}	LT_{99}	Slope	x^2	*p*	*df*
A. diaperinus	2.4 (1.9–2.8)	4.63 (4.1–5.7)	5.5 (4.8–7.0)	0.7 ± 0.1	25.33	0.444	25
T. molitor	1.8 (1.1–2.2)	3.6 (3.1–4.9)	4.3 (3.6–6.3)	0.9 ± 0.2	23.74	0.534	25

Table 2. Mean mortality (% ± SE) of adults of *A. diaperinus and T. molitor,* 7 and 14 days after the termination of the exposure to 3000 ppm of phosphine.

Species	7 Days	14 Days
A. diaperinus	20.0 ± 5.7 *	40.0 ± 6.6 *
T. molitor	60.0 ± 9.4	93.3 ± 3.3
t	−3.61	7.15
p	0.002	<0.001

* Means with asterisks, obtained on *A. diaperinus*-exposed adults, are significantly different from the respective means, obtained on *T. molitor*-exposed adults, within each post-exposure period and column larvae, according to Students' *t*-test at $p < 0.05$.

3.3. Bioassay III

All adults of both species were immobilized after 90 min of exposure at 3000 ppm of phosphine. Regarding adults of *A. diaperinus*, a noticeable level of survival was observed, as the mortality recorded was 48.8 and 53.3%, respectively, for 7 and 14 days post-exposure. In contrast, for *T. molitor*, a 100% mortality was recorded for both post-exposure intervals (Table 3).

Table 3. Mean number (% ± SE) of immobilized adults of *A. diaperinus* and *T. molitor*, after 90 min of exposure to 3000 ppm of phosphine and mortality (% ± SE) of adults of the aforementioned species, after the termination of 7 and 14-day post-exposure periods.

Species	90 min	7 Days	14 Days
A. diaperinus	100.0 ± 0.0	48.8 ± 8.2 *	53.3 ± 10.5 *
T. molitor	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
t	-	−6.20	4.42
p	-	<0.001	<0.001

* Means with asterisks, obtained on *A. diaperinus*-exposed adults, are significantly different from the respective means, obtained on *T. molitor*-exposed adults, within each post-exposure period and column larvae, according to Students' *t*-test at $p < 0.05$.

4. Discussion

This research demonstrates the efficacy of phosphine for the control of *A. diaperinus* and *T. molitor*. *Alphitobius diaperinus* adults were found to be more tolerant than those of *T. molitor*, as noticeable survival was observed, even after 90 min of exposure to 3000 ppm, as well as at the 7- and 14-day post-exposure intervals, whereas 100% mortality was noted for *T. molitor*. In an earlier study, Athanassiou et al. [20] used the PTT protocol to test different populations of major stored product beetle species, evaluating the knockdown time as a "threshold" to separate susceptible from tolerant populations. The times to knockdown here are comparable with those of the species which are listed in the Athanassiou et al. [20] study for both species tested. In addition, the post-exposure mortality times used here suggest that there is considerable delayed mortality, a fact that should be taken into account in "real world" fumigations in the case of the occurrence of surviving individuals. For *T. castaneum*, it was found that delayed mortality is likely to occur in the case of individuals that are susceptible to phosphine, while resistant populations exhibit a rapid post-exposure recovery [20].

The dose response protocol has long been regarded as a major diagnostic for resistance to phosphine and has been used with good results in different stored product insect species [6,16,18]. Based on our results, for both species tested, all life stages were found to be susceptible to phosphine, as mortality was complete (100%) even at the lowest concentration of 50 ppm for 3 days, whereas exposure to 100 ppm for the same interval was sufficient to kill all the eggs. As expected, eggs of both *A. diaperinus* and *T. molitor* were more tolerant to phosphine in comparison with the other life stages, which is in accordance with what has been noted for other stored product insect species [2,5,6,17,29]. For example, Gourgouta et al. [30] found that eggs of the khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) are by far the most tolerant life stage, as compared with the other life stages of this species, including its diapausing larvae. In this study, the authors reported that to obtain 100% egg mortality after 3 days of exposure, 1000 ppm of phosphine was required, in contrast with the other life stages where 50 ppm was found to be sufficient [30]. Furthermore, Athanassiou et al. [20] examined the efficacy of phosphine for different life stages of *T. inclusum* and *D. maculatus* and recorded that eggs of both of these species required considerably higher levels of phosphine to obtain 100% mortality, as compared with all other life stages. Despite the fact that our results here are in agreement with the previous observations for the reduced susceptibility of eggs to phosphine, the eggs of both *T. molitor* and *A. diaperinus* were only two times more tolerant than the other life stages, suggesting that phosphine can be used with success for the control of these two

species without the need to reach concentrations that are extremely high, as in the case of *T. granarium* [30]. As both species are usually not the dominant beetle species found in storage and processing facilities, they are often not treated with phosphine, and hence, the possibilities of resistance development can be considered as lower than for dominant species such as *T. castaneum* [31].

To our knowledge, the available published data for the efficacy of phosphine against *A. diaperinus* are based on the quantification of treatments from field tests, which were carried out only to estimate control levels without the use of resistance evaluation protocols [28,32]. Essentially, this is the first research indicating the effect of phosphine on different life stages of this species using standard laboratory exposure protocols. Since, traditionally, the control of this species in poultry farms is primarily achieved with the application of residual contact insecticides on the walls and floor, most of the research data concern pyrethroids and organophosphates [23,24,33–36]. Most of the active ingredients that have been tested as contact insecticides against this species have been found to be effective, but there are cases where resistance has been recorded [23,35,37,38]. Likewise, the majority of the data that are available for the control of *T. molitor* are based on the utilization of contact insecticides, with some being more effective than others [38,39]. For instance, larvae of this species are tolerant to diatomaceous earths at concentrations that are lethal for other major stored product beetle species [40–46]. Nevertheless, this is the first work that has examined the efficacy of phosphine against this species.

Our study adds important information regarding the control of *A. diaperinus* and *T. molitor*, underlining the high effectiveness of phosphine in all the life stages of both species. In addition, we provide information that can be further evaluated for inclusion in the PTT kit as a rapid diagnostic for tolerance to phosphine for these two species. As both species are now important sources of food and feed and are massively reared for this purpose in different parts of the world, control methods are an important element in this production procedure to minimize cross infestations in commercial rearing units, which are becoming more common [46]. Nevertheless, we consider that the results of the present study are mostly related to protocols that can be applied for the control of these two species at the post-harvest stages of durable agricultural commodities, rather than in insect rearing units, as gases will also eliminate the "beneficial" *A. diaperinus* and *T. molitor* individuals.

Author Contributions: Conceptualization, C.G.A.; methodology, M.G. and C.G.A.; investigation, M.G.; resources, C.G.A.; data curation, M.G.; writing—original draft preparation, M.G.; writing—review and editing, C.G.A.; visualization, M.G. and C.G.A.; supervision, C.G.A.; project administration, C.G.A.; funding acquisition, C.G.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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