Predatory Abilities of The Backswimmer *Buenoa Amnigenus* Are Not Impaired After Sublethal Exposures To Pyriproxyfen

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Abstract

Pyriproxyfen is a juvenile hormone analogue that is commonly used to control the immature stages of mosquitoes in both artificial and natural water reservoirs. Recently, concerns have been raised regarding the pyriproxyfen community effectiveness in preventing vector-transmitted diseases. Such concerns have been based on the unintended effects on non-target organisms and selection of resistant mosquito populations. Thus, this investigation was conducted aiming to evaluate the toxicity of pyriproxyfen to *Aedes aegypti* (Diptera: Culicidae) larvae and the backswimmer *Buenoa amnigenus* (Hemiptera: Notonectidae), a naturally occurring mosquito larvae predator. We also assessed the abilities of backswimmers exposed to sublethal levels of pyriproxyfen to prey upon mosquito larvae (L2) under three larval densities (3, 6, or 9 larvae/100 mL of water) using artificial containers. Our results revealed that pyriproxyfen killed backswimmers only at concentrations higher than 100 mg active ingredient [a.i.]/L, which is 10 times higher than that recommended for larvicidal field application (i.e, 10 mg a.i./L). Interestingly, the abilities of backswimmers exposed to sublethal levels of pyriproxyfen (100 mg a.i./L) to prey upon mosquito larvae were not affected. Harmful effects on the backswimmer predatory abilities were detected only at concentrations of 150 mg a.i./L and when there was a higher prey availability (i.e., 9 larvae/100 mL of water). Together, our findings indicate that the reduced community effectiveness of this insecticide derives from factors other than its detrimental effects on non-target organisms such as the backswimmers.

Introduction

The use of larvicidal insecticides to control mosquitoes that transmit human diseases such as Zika, dengue fever, and chikungunya has proven to be very successful (Darriet et al. 2010; Ohba et al. 2013; Maoz et al. 2017; Marina et al. 2018; Roiz et al. 2018; WHO 2020). Pyriproxyfen, the most used larvicide in both artificial and natural water reservoirs (Darriet et al. 2010; Ohba et al. 2013; WHO 2013), is a mimic of the natural juvenile hormone which can disrupt embryonic and larval insect development and impede the adult emergence, leading to an effectively reduction on mosquito populations (Sullivan and Goh 2008). However, previous studies have reported that the harmful effects of environmentally realistic concentrations of pyriproxyfen to non-target organisms (Devillers 2020; Moura and Souza-Santos 2020).

The unintended exposures to pyriproxyfen of non-target organisms (e.g., larvivorous fishes and microcrustaceans) that inhabit the same breeding sites as mosquitoes can lead to reduced locomotion in a number of species and have raised concerns regarding its low toxicity (Caixeta et al. 2016; Truong et al. 2016; Vieira Santos et al. 2017). The increasing number of reports demonstrating such pyriproxyfen unintended effects has affected the expectations regarding these molecules in terms of their community effectiveness, selection of resistant mosquito populations, and detrimental effects on non-target aquatic invertebrates (Maoz et al. 2017; Vieira Santos et al. 2017; Devillers 2020; Valbon et al. 2021), which may impact the ecological services provided by naturally occurring aquatic predators of mosquito larvae (Antwi and Reddy 2015; Valbon et al. 2018).

Among the aquatic insects endangered by unintended pyriproxyfen exposure, the semiaquatic backswimmers (Hemiptera: Notonectidae) represent an interesting model organism, as these insects' function not only as mosquito predators, but also as prey of vertebrates and other invertebrates, contributing to shape community structure (Van de Meutter et al. 2008; Shaalan and Canyon 2009; Secondi and Raux 2020). Backswimmers are cosmopolitan predators and are among the first insects to colonize freshwater habitats, such as artificial and natural water reservoirs (Giller and McNeill 1981), which certainly increase their probability of facing insecticide-contaminated ecosystems. Both nymph and adult backswimmers are primarily sit-and-wait surface predators (i.e., ambush strategy), that can also actively explore the water column using their forelegs to rapidly capture prey (Giller and McNeill 1981; Gutiérrez et al. 2017b).

Recent studies have suggested the use of aquatic insects as biological agents for controlling mosquito larvae (including *Aedes* spp.) in confined and natural environments (Shaalan and Canyon 2009; Sivagnaname 2009; Kweka et al. 2011; Eba et al. 2021). For instance, backswimmers were the most aggressive predator on *Anopheles* (Diptera: Culicidae) mosquito larvae when compared to dragonflies (Odonata: Libellulidae) and water bugs (Hemiptera: Belostomatidae) (Eba et al. 2021). Thus, biological control agents might be an alternative not only to be preserved/employed into integrated mosquito management but also combined with insecticide (Roiz et al. 2018). However, whether pyriproxyfen would cause detrimental effects on predatory of backswimmers remains poorly understood.

Here, we hypothesized that pyriproxyfen has toxic effects on backswimmers. Further, sublethal pyriproxyfen exposure can alter the predatory abilities of backswimmers to prey upon mosquito larvae. This insecticide/predator/prey interactions may represent a threat to backswimmers, a non-target aquatic insects capable of coexisting in mosquito breeding sites (Arredondo-Jimenez and Valdez-Delgado 2006; Shaalan and Canyon 2009). We took toxicological and behavioral approaches to investigate the impact of pyriproxyfen on the backswimmer *Buenoa amnigenus* (Hemiptera: Notonectidae). We used yellow fever mosquito *Aedes aegypti* (Diptera: Culicidae) as the model organism because of its widespread presence in simple aquatic communities and the potential to be preyed upon by generalist predators (Shaalan and Canyon 2009; Sivagnaname 2009). Specifically, we evaluated (1) whether pyriproxyfen caused similar mortality in *A. aegypti* larvae (target organism) and *B. amnigenus* adults (non-target organism), (2) whether *B. amnigenus* adults recovered from the exposure to sublethal levels of pyriproxyfen, and (3) whether backswimmer adults exposed to sublethal levels of pyriproxyfen showed impaired performance to prey upon mosquito larvae at different prey densities. Our findings suggest that pyriproxyfen is more toxic to *A. aegypti* larvae than *B. amnigenus* adults. Harmful effects on predatory behavior of *B. amnigenus* were observed only at high concentration of pyriproxyfen (150 µg a.i./L) and when these predators faced high larvae availability.

Materials And Methods

Insects

We used second instar *A. aegypti* larvae (L2) of an insecticide-susceptible strain (PPCampos, originally collected at Campos dos Goytacazes, Rio de Janeiro State, Brazil) that had been maintained under controlled conditions (temperature: 25 ± 2°C, relative humidity: 60 ± 2 %, and photoperiod of 12h of light), as previously described (Haddi et al. 2017; Mendes et al. 2017). Adult of backswimmers, *B. amnigenus*, were collected from fish farming installations at the Federal University of Viçosa (UFV, Viçosa, MG, Brazil, 20°45' S, 42°52' W). Before experimental analysis, groups of 50 backswimmers were maintained in glass beakers containing 500 mL of dechlorinated tap water (conductivity of 425 ± 30.5 µS/cm and pH of 7.4 ± 0.1) for 24 h under controlled conditions (25 ± 2°C, 12 h of photophase) (Gutiérrez et al. 2017a). Identification of male specimens of *Buenoa* was based on Truxal's key (Truxal 1952), using a stereomicroscope. Specimens were anaesthetized with ice before identification.

Toxicological bioassays

We examined the susceptibility of *A. aegypti* larvae and *B. amnigenus* adults to pyriproxyfen [100 g active ingredient (a.i.)/L, emulsifiable concentrate, Sumitomo Chemical Ltda, São Paulo, Brazil] at different nominal concentrations (ranging from 10 µg a.i./L to 500 µg a.i./L). We first exposed *A. aegypti* larvae to concentrations of pyriproxyfen recommended for their control in field applications (i.e., 10 and 20 µg a.i./L). Further, to investigate the selectivity of pyriproxyfen we exposed backswimmer adults to the concentrations of 100 and 150 µg a.i./L, which is 10 and 15 times higher than that recommended for *A. aegypti* larvae control. For survival bioassays, groups of 25 mosquito larvae (L2) were transferred to glass beakers containing 100 mL of insecticide, while groups of 10 adult backswimmers were placed into glass beakers containing 300 mL of insecticide. All beakers were covered with organza fabric to prevent insect escape. For each insecticide concentration treatment, we used 100 mosquito larvae, or 40 backswimmers. Mortality was assessed every 24 h for 10 consecutive days under pyriproxyfen solution (i.e., without insecticide recovery) and insects that remained motionless after being repeatedly stimulated mechanically with a pipette were considered dead. In control groups, we exposed all insects to dechlorinated tap water (conductivity of 425 ± 30.5 µS/cm and pH of 7.4 ± 0.1). For the concentration-mortality bioassays with backswimmers, we used the same protocol described above with slight alterations, in which 4 replicates (groups of 10 insects) were used for each concentration and mortality was assessed only once (after 24 h of exposure) (Gutiérrez et al. 2017a). In order to assess the ability of backswimmer to recover from pyriproxyfen sublethal exposure, we conducted a survival bioassay (as above described) with individuals that were previously exposed for 24h to pyriproxyfen at concentrations of 100 and 150 µg a.i./L, which was unexpectedly the same sublethal concentrations LC_1 and LC_{10} estimated by concentration-mortality analysis. After 24h of pyriproxyfen exposure, backswimmers were transferred to dechlorinated tap water and mortality was assessed every 24 h for 10 consecutive days (with insecticide recovery). All toxicological bioassays were performed at controlled temperature (25 ± 2°C), humidity (60 ± 2 %) and photoperiod (12h of light phase) conditions.

Predation bioassays with backswimmer adults

To assess the abilities of backswimmers exposed to sublethal levels of pyriproxyfen to prey upon *A. aegypti* larvae (L2), adults were exposed to pyriproxyfen at concentrations of 100 µg a.i./L (lethal

concentration; LC_1) and 150 µg a.i./L (LC_{10}). After 24 h of pyriproxyfen exposure, backswimmers were individually transferred into glass vials containing 100 mL of dechlorinated tap water and left for 1 h to acclimatize. Subsequently, we recorded the number of L2 larvae preyed upon by backswimmers that were individually exposed to one of three larval densities (3, 6, or 9 larvae/100 mL of dechlorinated tap water). At least seven replicates (i.e., backswimmer) were used for each combination of insecticide concentration and prey density. For each density, the larvae were transferred using a Pasteur pipette without injuring them. The number of preyed larvae (i.e., larvae carcasses floating on the water surface or in the bottom of the vial) was evaluated at 20 min intervals for 2 h just after the exposure period (24h), and the larval density re-established at each evaluation (Gutiérrez et al. 2017a). All predation bioassays were performed at controlled temperature (25 ± 2°C), humidity (60 ± 2 %) and photoperiod (12h of light phase) conditions.

Statistical analysis

For the survival bioassay (mosquito larvae and backswimmer adults), we applied Kaplan-Meier estimators (Log-rank method) available in SigmaPlot 12.0 (Systat Software, San Jose, California, USA). We compared survival curves using the Holm-Sidak's method. Concentration–mortality data were subjected to probit analysis (SAS Institute 2008). For the larval predation data, we used a repeated measures analysis of variance to determine the effects of insecticides, larval density, and recovery time using SAS software (SAS Institute 2008). The number of preyed larvae in each 20 min interval on the first day after exposure were used as replicates (within-sample variation) to avoid problems associated with temporal pseudo-replication (von Ende 1993; Paine 1996). The total daily number of mosquito larvae consumed was subjected to one-way analyses of variance (ANOVA) (SAS Institute 2008). We assessed the assumptions of normality and homogeneity of variance using the UNIVARIATE procedure (SAS Institute 2008), and no data transformations were necessary.

Results

Toxicity of pyriproxyfen to Aedes aegypti **larvae and** Buenoa amnigenus **adults**

Survival analyses showed significant differences between the pyriproxyfen concentrations for mosquito larvae (Log-Rank: $\chi^2 = 96.01$, $df = 2$, $P < 0.001$) (Fig. 1A) and backswimmer adults (Log-Rank: $\chi^2 = 40.17$, $df = 2$, $P < 0.001$) (Fig. 1B). The median lethal time (i.e., LT_{50}) for larvae exposed to the concentration of pyriproxyfen recommended for mosquito control (i.e., 10 µg a.i./L) was 48 h. A similar median lethal time for backswimmers was observed only at concentrations as high as 100 µg a.i./L, indicating that pyriproxyfen is approximately 10-fold more toxic to mosquito than to backswimmers. The concentration-mortality results for pyriproxyfen in backswimmers were satisfactorily fitted by a probit model ($\chi2 = 2.46$; $df = 4$, $P = 0.65$), which allowed the estimation of a median lethal concentration (LC50) of 232.4 (205.4–255.6) µg a.i./L (Fig. 1C, Table 1). We also estimated the LC1 and LC10 values (100.0 [68.8–135.3] and 150.0 [114.0–177.6] µg a.i./L, respectively) for backswimmers. We further exposed backswimmer adults to a single pulse of sublethal levels of pyriproxyfen (i.e., LC_1 and LC_{10}) for 24h. All individuals survived from pyriproxyfen sublethal exposure and then were transferred to dechlorinated tap water where the

survival ability was assessed. Survival analyses did not show significant differences between the pyriproxyfen concentrations and control for backswimmer adults (Log-Rank: $\chi^2 = 3.869$, $df = 2$, $P = 0.144$) (Fig. 1C). Backswimmers survived 3-fold longer (i.e., LT = 144h) than individuals that did not have the chance of recovery from pyriproxyfen exposure (i.e., LT = 48h). We also exposed individuals to the LC1 and LC10 concentrations to evaluate the potential sublethal effects of pyriproxyfen on the ability of backswimmers to prey upon mosquito larvae.

Table 1. Analysis of variance with repeated measures over time for the mean number of *A. aegypti* larvae preyed upon (at 20 min intervals) by *B. amnigenus* after 24 h of exposure to pyriproxyfen (100 or 150 mg a.i./L).

Sources of variation	df	F	*P*		
Between factors					
Insecticide (I)	2	2.06	0.1564		
Density (D)	2	0.78	0.0531		
I × D	4	1.74	0.1517		
Error	62	-	-		
	df_{num}	df_{den}	Wilks' lambda	F	*P*
Within each factor					
Time (T)	5	8	0.614	7.29	<0.0001**
T × I	10	116	0.854	0.95	0.4942
T × D	10	116	0.497	4.86	<0.0001**
T × I × D	20	193	0.523	2.08	0.0059*

Effects of pyriproxyfen on the predatory abilities of Buenoa amnigenus adults

A repeated measure ANOVA revealed that neither pyriproxyfen exposure nor prey density affected the predatory abilities of *B. amnigenus* adults (Table 1, Fig. 3). Results, however, revealed significant effects for time ($F(5,6) = 7.29$, $P < 0.0001$), the interaction between time and density ($F(10,116) = 4.86$, $P < 0.0001$), as well as for the interaction between time, pyriproxyfen exposure, and density ($F(20,193 = 2.08$, $P = 0.0059$). No significant effects were found for the interaction between time and pyriproxyfen exposure ($F(20,193) = 2.08$, $P = 0.49$) (Table 1, Fig. 3). At the lowest prey densities (i.e., 3 *A. aegypti* larvae/100 mL of water), individual backswimmers that survived sublethal pyriproxyfen exposure (150 µg a.i./L) preyed upon a greater number of mosquito larvae at the first evaluation, reducing their predatory performance over time (Fig. 3A), which resulted in non-significant differences for the total number of mosquito larvae preyed upon when compared to those recorded for backswimmers not exposed to pyriproxyfen (Fig. 4A).

A similar pattern was recorded for the experiment with the intermediary number of larvae (i.e., 6 mosquito larvae/100 mL of water), with the difference being a change recorded for the backswimmers that survived the lowest sublethal exposure to pyriproxyfen (100 µg a.i./L) (Fig. 3B). When backswimmer adults were offered the highest mosquito larva density, however, insects exposed to the highest pyriproxyfen concentration (i.e., 150 µg a.i./L) showed a reduced predatory ability when compared to unexposed insects (Fig. 3C). It is worth noting that backswimmer adults not exposed to pyriproxyfen preyed voraciously upon of mosquito larvae, eating up to 40 larvae in less than 2 h (Fig. 4C).

Discussion

Here, we demonstrated that *B. amnigenus* adults voraciously preyed upon mosquito larvae, especially when the prey was at higher densities. Such findings reinforce previous reports that indicates backswimmers as promising biological agents to prey upon and control mosquito larvae population of different species (Shaalan and Canyon 2009; Gutiérrez et al. 2017a; Eba et al. 2021). Here, we also demonstrated that pyriproxyfen was more toxic to *A. aegypti* larvae than to backswimmers. Interestingly, our results revealed that the abilities of adult backswimmers to prey upon mosquito larvae were unaffected by pyriproxyfen exposure, even at concentrations as high as 100 µg a.i./L, which is a 10-fold higher concentration of pyriproxyfen than recommended for larvicidal field applications. Even though some studies have shown the potential of mosquito larvae to encounter their predator in artificial and natural environments (Shaalan and Canyon 2009; Sivagnaname 2009; Kweka et al. 2011; Eba et al. 2021), it is worth noting that we used *A. aegypti* larvae focusing on a prey/predator association model to test our hypothesis. Thus, such scenario might not necessarily reflect the real interactions in the aquatic ecosystems.

Although the insecticide selectivity has recently been investigated in different aquatic invertebrate species (Ser and Cetin 2015; Gutiérrez et al. 2016; Santos et al. 2018; Valbon et al. 2018; Reegan et al. 2020), it remained unclear whether pyriproxyfen could impact the survival and predatory abilities of backswimmers. Our results demonstrated differential susceptibilities to pyriproxyfen between the mosquito larvae and backswimmer adults, which can be explained in different ways. Firstly, given that the primary mode of action of pyriproxyfen is disrupting the insect endocrine system, maintaining the insect in an immature state until their death or reducing the number of emerged adults (Sullivan and Goh 2008; Ginjupalli and Baldwin 2013), it is reasonable to consider that the pyriproxyfen binding site receptors are more promptly available in the second instar mosquito larvae than backswimmer adults. Indeed, in a recent investigation we could show that low pyriproxyfen concentration (i.e., 2.5 µg a.i./L) reduced the survival and predatory abilities of nymphs of the water bug *Belostoma anurum* (Hemiptera: Belostomatidae), a naturally occurring mosquito control agent in the Neotropical region (Valbon et al. 2019a, 2021).

Our findings may not suffice to rule out the possibility that *B. amnigenus* higher tolerance to pyriproxyfen by having differential molecular interactions between pyriproxyfen and their receptor site receptors. However, the results described here are sufficient to suggest that backswimmers tolerance likely reflects

their physiological status and life stage as demonstrate to other predator-prey relationships. For instance, differences in the properties of the cuticle (e.g., thickness and binding proteins) (Wood et al., 2010) and insecticide metabolism by detoxifying enzymes (e.g., cytochrome P450 monooxygenases, glutathione-S-transferase, and general esterases) of backswimmers and mosquito larvae could also contribute to explain the pyriproxyfen selectivity as described for other aquatic insects (Liu 2015; Valbon et al. 2019b).

Because backswimmers are cosmopolitan predators and are among the first insects to colonize freshwater habitats (Giller and McNeill 1981), they are constantly prone to reach insecticide-contaminated ecosystems in both artificial and natural water reservoirs. However, as notonectid adults are well-flight insects (i.e., semi-aquatic organisms), these organisms can easily evade contaminated habitats migrating to more favorable environments (Briers and Warren 2000; McCauley and Rowe 2010). Our results for the survival abilities of *B. amnigenus* under such scenario, i.e., facing a 24h pyriproxyfen exposure and placed under non-contaminated environment, revealed that backswimmers did not show any difference in their survival abilities when compared to those pyriproxyfen-unexposed predators.

Recent studies have reported that mosquito larvicides can impair key behavioral parameters in aquatic predators, such as locomotion and prey catching, leading to unsuccessful foraging (Gutiérrez et al. 2017b; Valbon et al. 2018; Lajmanovich et al. 2019). Interestingly, at low and intermediate prey densities, backswimmers that were previously exposed to pyriproxyfen preyed on a higher number of mosquito larvae than unexposed insects (i.e., soon after pyriproxyfen exposure). A similar foraging pattern has been reported in a coexisting backswimmer, *Buenoa tarsalis*, when sublethally exposed to *Bacillus thuringiensis* var *israelensis* (Bti) toxins-based larvicide (Gutiérrez et al. 2017a), suggesting that backswimmers may share hormetic-like responses (i.e., when an unexpected beneficial effect occur in individuals facing non-lethal stresses, (Guedes et al. 2017)) in insecticide-contaminated environments. However, as we found no alteration in the total number of larvae preyed upon by backswimmers exposed to sublethal levels of pyriproxyfen, at both mosquito larval densities, further studies are required before drawing firm conclusions. At the highest prey density, we found that backswimmers exposed to high pyriproxyfen concentrations (150 µg a.i./L, a concentration 15-fold higher than that recommended for field application) preyed upon significantly fewer mosquito larvae than unexposed predators.

Conclusion

The management of mosquito vectored diseases needs to be tailored by different entomological epidemiological risk scenarios and needs to consider the multitude of factors (e.g., global trade, international travel, urbanization, water storage practices, lack of resources for intervention) that may affect its implementation (Maoz et al. 2017; Roiz et al. 2018). Given its efficiency in reducing mosquito populations at low concentrations without impairing the abilities of backswimmers to predate upon mosquito larvae, our findings suggest that pyriproxyfen-exposed aquatic ecosystems may not offer higher risks to backswimmer populations and the suggested lack of community effectiveness following the use of pyriproxyfen to control vector transmitted diseases may lie elsewhere. However, given that pyriproxyfen disrupts insect immature stage from developing into adulthood, to increase the

understanding of the impact of this pesticide in both prey and predator further experiments aiming to evaluate transgenerational effects will help to produce a better insight into such sublethal effects in these naturally occurring mosquito larvae predators.

Declarations

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Authors' contributions

W.V.:Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing**.** S.H.C.A.: Investigation, Formal analysis, Writing - review & editing. R.S.N.: Investigation, Formal analysis**.** J.F.B.:Identified the insect species,Writing – review & editing.P.L.N.:Funding acquisition, Supervision, Writing - review & editing.E.E.O.:Conceptualization, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing**.**

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Compliance with ethical standards

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Figures

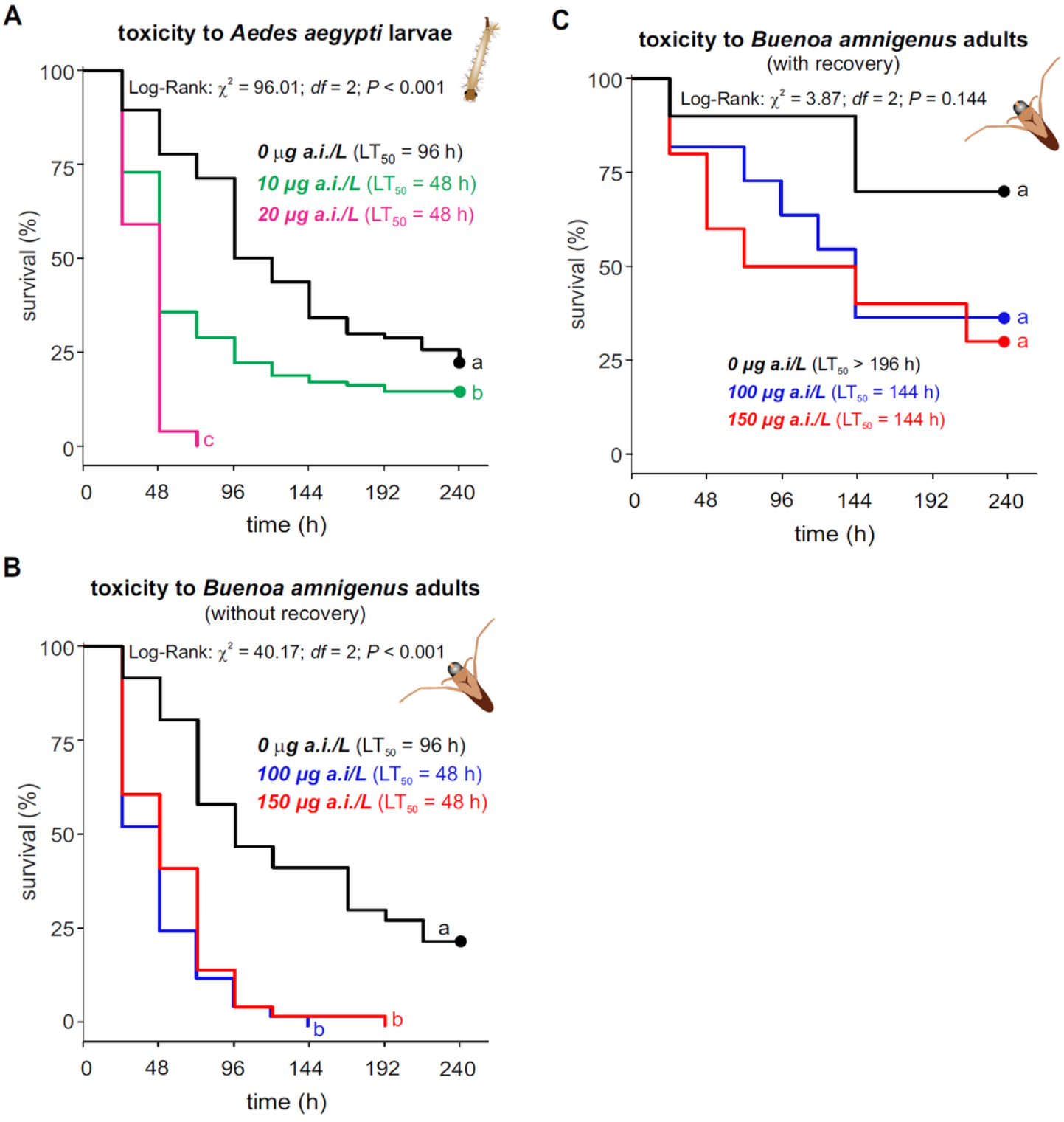

Figure 1

Toxicity of pyriproxyfen to Aedes aegypti larvae and backswimmer Buenoa amnigenus adults. Survival curves of mosquito larvae (A) and backswimmer adults (B) exposed to different concentrations of pyriproxyfen for 10 consecutive days (without insecticide recovery). (C) Survival curves of backswimmer

adults exposed to different concentrations of pyriproxyfen for 24h and then transferred to insecticide-untreated water (with insecticide recovery). Treatments with the same letter do not differ according to Holm-Sidak's test (P > 0.05).

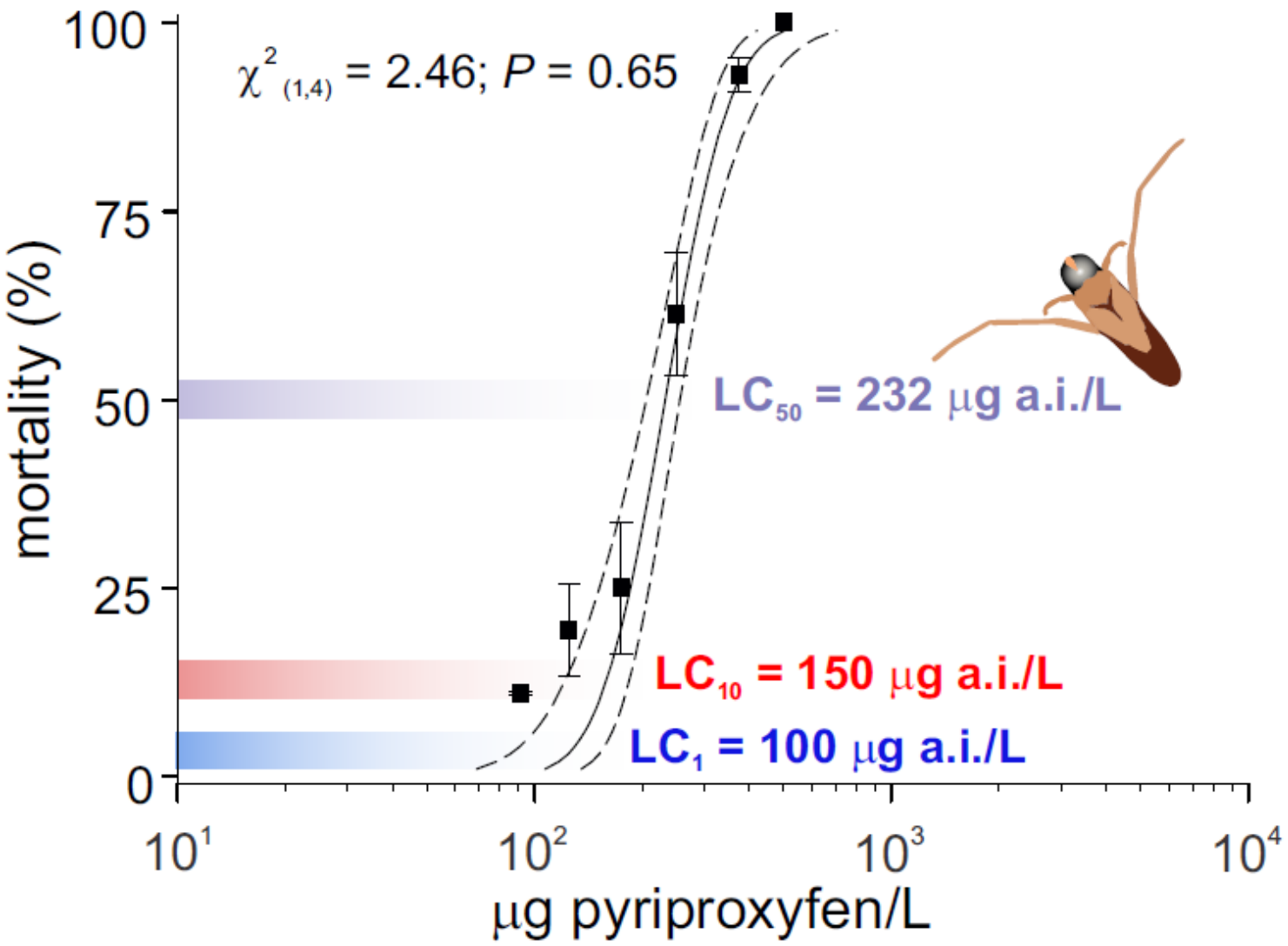

Figure 2

Concentration-response curve of pyriproxyfen for backswimmer adults after 24h of exposure. The lines denote the lethal concentration (LC) values based on concentration-mortality bioassays using probit analyses. Dotted lines represent 95% confidence intervals for the LC estimations. Symbols (± standard error) show the average mortality recorded for each insecticide concentration.

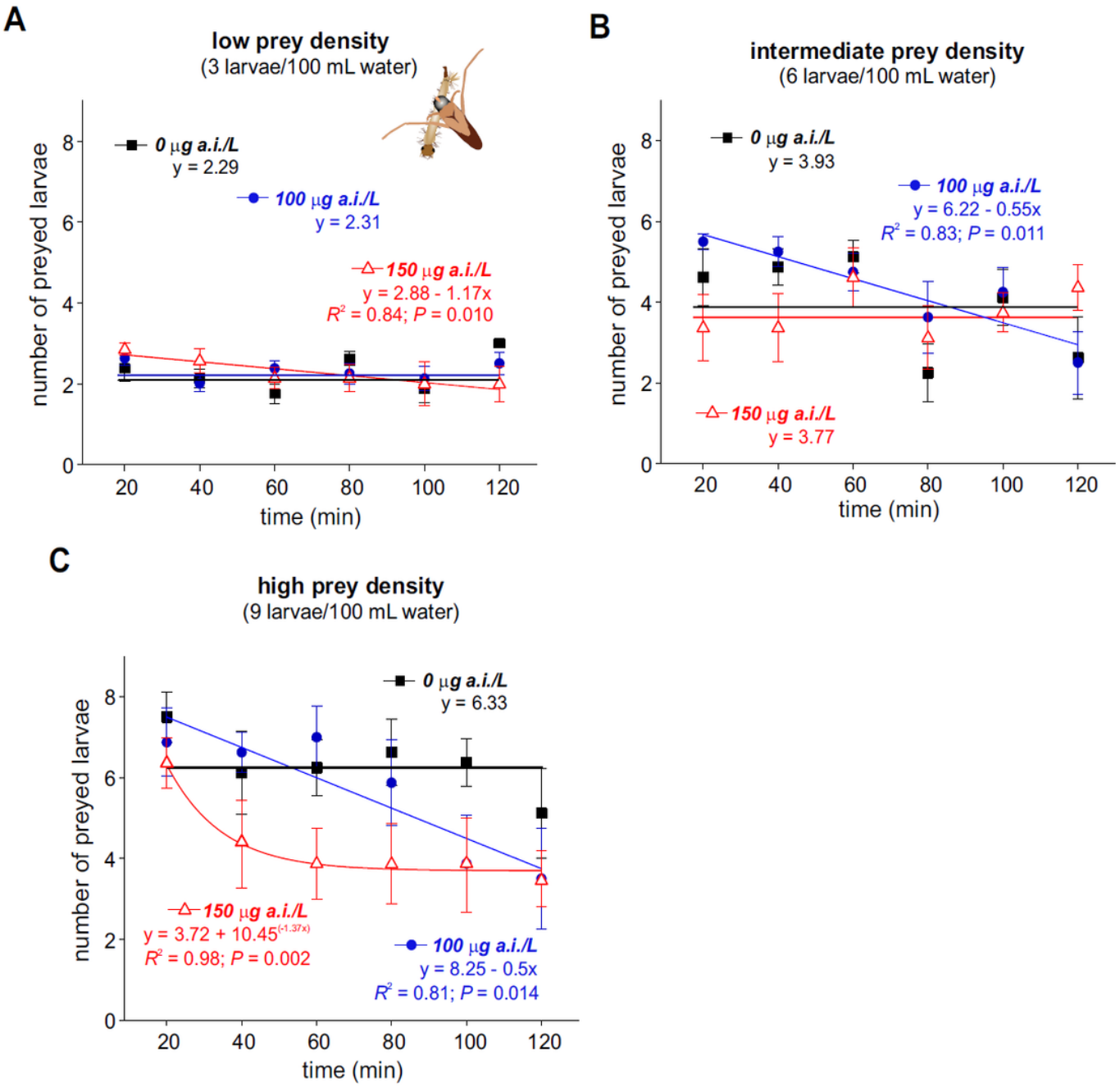

Figure 3

Abilities of the backswimmer Buenoa amnigenus to prey upon Aedes aegypti larvae. The predatory abilities of backswimmers that faced sublethal exposure to pyriproxyfen (either 100 or 150 µg a.i./L), or were unexposed, were recorded when these predators were subjected to larval densities of three (A), six (B), and nine (C) larvae/100 mL water. Symbols show the average number (± standard error, SE) of preyed larvae by each backswimmer.

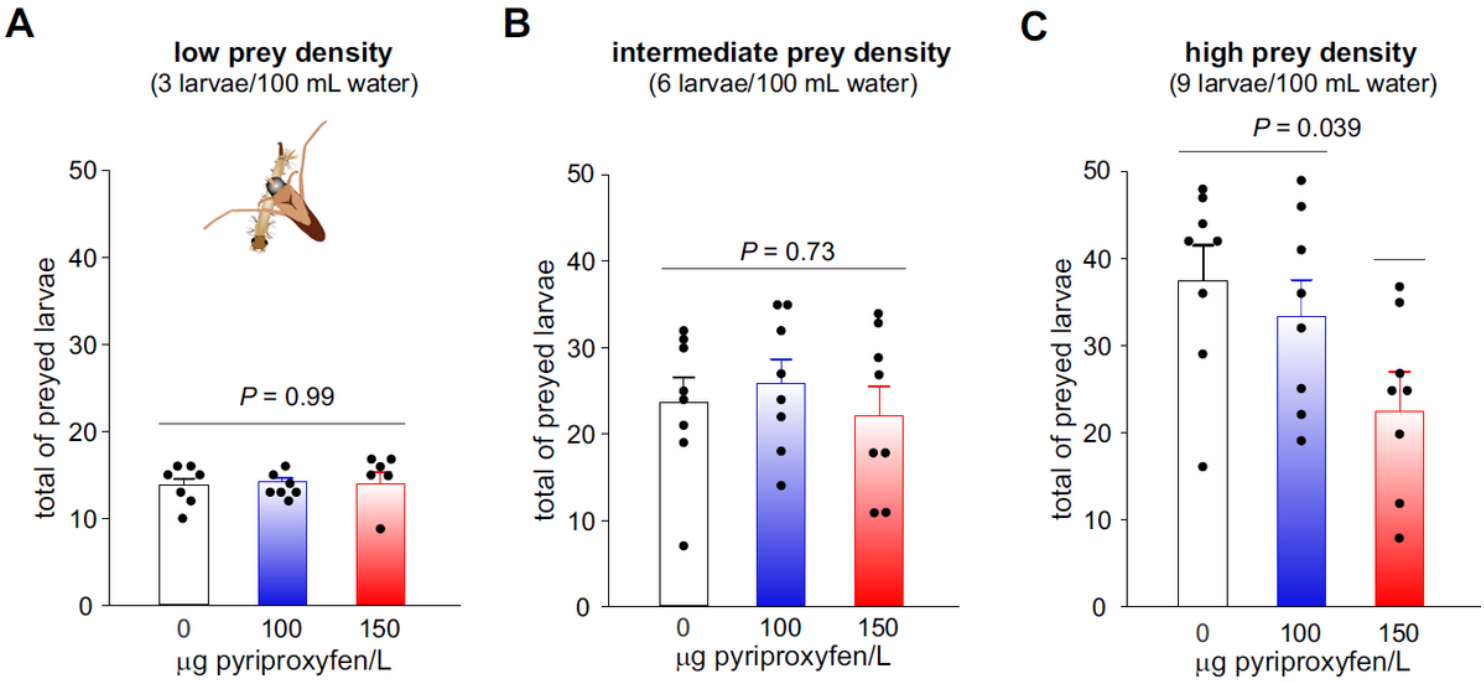

Figure 4

The total number of Aedes aegypti larvae preyed upon by backswimmers Buenoa amnigenus. The predators' abilities were assessed at larval densities of three (A), six (B) and nine (C) larvae/100 mL of water. The results represent the average number (± standard error, SE) and dots denote the value of each replicate. Means grouped under the same horizontal line are not significantly different by Tukey's HSD test ($P > 0.05$).