Toxicity of the Jaburetox Peptide to the Multi-Host Insect-Pest Helicoverpa armigera (Lepidoptera: Noctuidae) Larvae

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Toxicity of the Jaburetox peptide to the multi-host insect-pest *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae

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Abstract

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) causes extensive damage to crops. The entomotoxic peptides fragments from Jack bean urease, Jaburetox and its truncated version Jaburetox Δ-β, present potential for controlling some insect species, and could provide an alternative for pest insect control in agricultural crops. This paper evaluated the effectiveness of these 2 peptides on consumption by and survival of the *H. armigera* larvae in 2 different instars of the larval stage. Neonates were fed leaves for 8 d with 2 peptide topical leaf treatments: (T1) 16 µg of Jaburetox, (T2) 16 µg of Jaburetox Δ-β; and 2 control treatments: (T3) sodium phosphate buffer, and (T4) distilled water. Leaves coated with either of the peptides induced higher mortality than the controls. The Jaburetox Δ-β induced the greatest mortality during the first d of feeding, but after 6 d both peptides were effective equally and caused about 75% mortality. Both peptide versions caused a delay in larval development, but the larger peptide caused a greater reduction in feeding. In a second experiment, third instar larvae were fed 1 of 2 treatments for 9 d: (T1) 80 µg of Jaburetox, and (T2) buffer control. Jaburetox treatment induced a delay in the larval development and a significantly higher mortality than the control. By 9 d, Jaburetox treatment caused 100% mortality. These results support further evaluation of the use of Jaburetox peptide in control strategies for *H. armigera*, including transgenic expression of this peptide in crops plants.

Key Words: urease derived peptide; insect control; entomotoxin; larval stage; transgenic plants

Resumo

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) causa danos extensos às culturas. Peptídeos entomotóxicos derivados de ureases de feijão-de-porco, Jaburetox e sua versão truncada Jaburetox Δ-β, apresentam potencial para controlar algumas espécies de insetos, e podem fornecer uma alternativa para o controle de insetos pragas em culturas agrícolas. Este trabalho avaliou a eficácia desses 2 peptídeos no consumo e sobrevivência das larvas *H. armigera* em 2 diferentes estádio de desenvolvimento larval. Larvas recém-nascidas foram alimentados por 8 d com 2 tratamentos de peptídeos aplicados topicamente sobre discos foliares: (T1) 16 µg de Jaburetox, (T2) 16 µg de Jaburetox Δ-β; e 2 tratamentos controles: (T3) tampão de fosfato de sódio e (T4) água destilada. Folhas cobertas com qualquer um dos peptídeos induziram maior mortalidade do que os controles. O peptídeo Jaburetox Δ-β induziu a maior mortalidade durante os primeiros d da alimentação, mas após 6 d ambos os peptídeos foram igualmente eficazes e causaram cerca de 75% de mortalidade. Ambas as versões de peptídeos causaram um atraso no desenvolvimento larval, mas o peptídeo com a versão completa causou uma maior redução na alimentação. Em um segundo experimento, larvas de terceiro instar foram alimentadas com 1 dos 2 tratamentos por 9 d: (T1) 80 µg de Jaburetox e (T2) controle tampão. O tratamento de jaburetox induziu um atraso no desenvolvimento larval e uma mortalidade significativamente maior do que o controle. Em 9 d, o tratamento de Jaburetox causou 100% de mortalidade. Esses resultados suportam uma avaliação mais aprofundada do uso do peptídeo jaburetox em estratégias de controle para *H. armigera*, incluindo expressão transgênica deste peptídeo em plantas cultivadas.

Palavras Chaves: peptídeo derivado de urease; controle de insetos; entomotoxina; fase larval; plantas transgênicas

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a pest on various agricultural crops, including soybean (*Glycine max* [L.] Merr.; Fabaceae), maize (*Zea mays* L.; Poaceae), tomato (*Solanum lycopersicum* L.; Solanaceae), and cotton (*Gossypium hirsutum* L.; Malvaceae) (Moral-Garcia 2006; Specht et al. 2013; Mamta et al. 2016). In Brazil, it was first identified in 2013 (Czepak et al. 2013; Salvadori et al. 2013), and is considered one of the principal agricultural pests in the world, with larvae causing extensive annual losses in crop production (Lim et al. 2016).

Control of *H. armigera* relies generally on application of chemical insecticides which, if used inadequately, may lead to the selection

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of resistance in this insect-pest, and potentially may be damaging to the environment (Asokan et al. 2014). The use of genetically modified plants containing insect resistant genes, such as those genes encoding *Bacillus thuringiensis* Berliner (Bacillaceae) (Bt) toxins, is an environmentally sustainable alternative.

However, in the case of the Bt technology where it is used extensively worldwide, and often done so with inadequate use of refuge areas, the resulting high selection pressure may lead to development of resistance over yr of use (Resende et al. 2014). Therefore, the identification of other entomotoxic protein encoding genes is needed to alleviate the over-use of Bt toxins. The *Jaburetox* gene, derived from plant urease, encodes a peptide that could prove to be an effective Bt toxin alternative.

Ureases are multifunctional proteins that are produced naturally by plants. Functions including defense and conversion of environmental nitrogen to bioavailable compounds are attributed to them. As part of their defense function, they have been demonstrated to have insecticidal and antifungal activity (Stanisçuaski & Carlini 2012; Carlini & Ligabue-Braun 2015; Becker-Ritt et al. 2017). Urease internal proteolytic peptides, including Jaburetox, were shown to have insecticidal activity demonstrated to control various insect orders including Lepidoptera, Hemiptera, Diptera, and Blattodea as reported by Stanisçuaski et al. (2005), Mulinari et al. (2007), Tomazetto et al. (2007), Defferrari et al. (2011), Martinelli et al. (2014), Galvani et al. (2015), and Becker-Ritt et al. (2017).

Jaburetox represents the entomotoxic portion of the jack bean urease II protein (a urease isoform from *Canavalia ensiformis* [L.] DC; Fabaceae) that is released upon digestion of jack bean urease II protein by insect digestive proteases (Mulinari et al. 2007). A synthetic gene, Jbtx, encoding the Jaburetox peptide was constructed from the jack bean urease II protein cDNA sequence, and expressed in *Escherichia coli* (Migula) Castellani & Chalmers; Enterobacteriaceae) (Mulinari et al. 2007). This peptide has been demonstrated to have entomotoxic properties for human disease insect vectors such as *Rodnius prolixus* Stål, *Triatoma infestans* Klug (both Hemiptera: Reduviidae), and *Aedes aegypti* L. (Diptera: Culicidae) (Becker-Ritt et al. 2017), and plant insect-pest such as *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Mulinari et al. 2007), *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Carlini & Grossi-de-Sá 2002), *Diatraea saccharalis* (F.) (Lepidoptera; Pyralidae), and *Telchin licus* (Drury) (Lepidoptera: Castniidae) (Becker-Ritt et al. 2017).

Jaburetox's mode of action includes alterations in the excretion and nervous systems resulting in insect mortality (Stanisçuaski & Carlini 2012; Piovesan et al. 2014; Galvani et al. 2015; Fruttero et al. 2016, 2017). Transgenic crop plants expressing Jaburetox could be a new strategy for controlling insects (Becker-Ritt et al. 2017), including *H. armigera*, if insecticidal activity to this insect can be demonstrated.

The Jaburetox peptide contains 93 amino acids (Mulinari et al. 2007). In studies to establish structure versus biological activity relationships, the Jaburetox Δ-β peptide, with 79 amino acids, was obtained by removing the C-terminal portion of the molecule capable of forming a β-hairpin (Martinelli et al. 2014). The Jaburetox Δ-β peptide induced mortality equivalent to the full version Jaburetox in *R. prolixus* and *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) when injected. However, when offered orally to *R. prolixus*, toxicity results were less evident and more variable (Martinelli et al. 2014).

This study aimed to test whether the entomotoxic peptides Jaburetox and Jaburetox Δ-β, when supplied orally, affect the behavior and survival of the *H. armigera* larvae and whether there are differences in toxicity between these 2 peptides.

Materials and Methods

INSECT REARING AND TOXIN PURIFICATION

Helicoverpa armigera larvae, collected in Brazil, were reared and maintained on an artificial diet (Greene et al. 1976) at the University of Passo Fundo Entomology Laboratory, Passo Fundo, Brazil, until used. Recombinant entomotoxic peptides Jaburetox (93 amino acids) and its truncated version Jaburetox Δ-β (lacking the β-hairpin motif from 61–74 aa) were produced by the Toxic Proteins Laboratory of the Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil. The Jaburetox and Jaburetox Δ-β production and purification were carried out according to Martinelli et al. (2014). Briefly, the recombinant proteins with a 6-His tag (6 Histidine residues) on their C-terminal were purified from *E. coli* BL21 (DE3)-RIL cells transformed with the pET23a-Jaburetox and pET23a-Jaburetox-Δβ plasmids. Cells were cultivated in 15 mL LB medium with 100 µg mL^{-1} of ampicillin, and 40 µg mL^{-1} of chloramphenicol overnight at 37 °C and 150 rpm. The entire content was used to inoculate 1 L of auto induction medium (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, glycerol 5 g L^{-1}, $(NH_4)_2SO_4$ 3.3 g L^{-1}, KH_2PO_4 6.8 g L^{-1}, Na_2HPO_4 7.1 g L^{-1}, glucose 0.5 g L^{-1}, and lactose 2 g L^{-1}, with 100 µg mL^{-1} of ampicillin and 40 µg mL^{-1} of chloramphenicol), and cultivated at 37 °C, 150 rpm, until an absorbance, A_{600nm}, of approximately 0.7 was achieved. The induction conditions were overnight, 20 °C, and 150 rpm. After cultivation, the cells were centrifuged at 8,000 × g for 10 mins at 4 °C and resuspended in 30 mL of buffer A (Tris-HCl 50 mM, pH 7.5, 500 mM of NaCl, and 5 mM of imidazole) and sonicated for 20 cycles of 1 min each at a frequency of 99 Hz to lyse the cells. The supernatant was separated via centrifugation at 15,000 × g for 40 min at 4 °C and added to a Chelating Sepharose (GE Healthcare, Chicago, Illinois, USA) affinity column charged with Ni^{2+}, previously equilibrated with buffer A. The column was washed using 10 vol of buffer B (Tris-HCl 50 mM, pH 7.5, 500 mM of NaCl, and 50 mM of imidazole) and elution was carried out with buffer C (Tris-HCl 50 mM, pH 7.5, 500 mM of NaCl, and 200 mM of imidazole). No His-tag cleavage was performed after the purification.

The eluted Jaburetox fraction was further purified using size exclusion chromatography on a Hiload Superdex 200 26/60 (GE Healthcare, Chicago, Illinois, USA) prep grade column equilibrated with buffer D (sodium phosphate 50 mM, EDTA 1 mM, and 1 mM of tris (2-carboxietil) phosphine – TCEP), mounted on an Akta Purifier (GE Healthcare, Chicago, Illinois, USA) system. The chromatography was carried out at a flow of 2.5 mL min^{-1} and 5 mL fractions were collected. Sample purity was verified using 15% SDS-PAGE and the protein concentration was determined using the Bradford assay. The peptides were dialyzed with sodium phosphate buffer 20 mM, pH 7.5, and the Bradford assay was used again to determine protein concentration; the peptide was then diluted to concentrations indicated in the Bioassay section. Peptides were stored at −20 °C until used.

TOXIN BIOASSAYS

Two different bioassays were conducted at the University of Passo Fundo Plant Biotechnology Laboratory, Passo Fundo, Brazil: (1) toxicity of Jaburetox and Jaburetox Δ-β peptides to neonatal *H. armigera* larvae, and (2) toxicity of Jaburetox to third instar *H. armigera* larvae.

TOXICITY OF THE JABURETOX AND JABURETOX Δ-B PEPTIDES TO NEONATE *HELICOVERPA ARMIGERA* LARVAE

The neonate *H. armigera* larvae were subjected to 4 different treatments: (T1) 16 µg of Jaburetox applied on leaf discs, (T2) 16 µg of Jaburetox Δ-β on leaf discs, (T3) sodium phosphate dialyzing buffer, and (T4) distilled water, with T3 and T4 acting as the control treatments. The concentration of the peptide tested here is based on the amount previously determined to be sufficient to kill 100% of *S. frugiperda* larvae (Mulinari et al. 2007). The experimental design was completely randomized with 4 repetitions. Each repetition consisted of 15 Petri dishes (5.0 cm × 0.8 cm, lined with filter paper moistened with sterile water) each containing 1 larva, into which the treated maize leaf discs (Hi-II genotype) were supplied. The peptide solution was applied on top of the discs with a micropipette, which were dried at room temperature before being offered to the larvae. The plates were maintained in a chamber acclimatized to 25 ± 2 °C, relative humidity of 70 ± 10%, and a 12:12 h (L:D) photoperiod.

The larvae were supplied the 0.5 cm^2 leaf discs on d 0 and on d 2 (48 h apart), each containing 8 µg of the Jaburetox or Jaburetox Δ-β peptides, diluted in 7.3 µL of sodium phosphate buffer and 0.01% of Silwet® (PhytoTec Labs, Lenexa, Kansas, USA) (Surfactant), every 48 h, totaling 16 µg of purified peptides offered to each larva. For the control treatments, discs with 7.3 µL of the respective solutions were supplied with the same surfactant (0.01% Silwet®). After d 4, 1 cm^2 foliar discs were supplied, without the treatments, to all larvae and renewed every 48 h until d 8.

TOXICITY OF THE COMPLETE VERSION OF JABURETOX TO THIRD INSTAR *HELICOVERPA ARMIGERA* LARVAE

The third instar larvae were reared on artificial diet (Greene et al. 1976) with a 12:12 h (L:D) photoperiod, at 25 ± 2 °C and 70 ± 10% relative humidity. These larvae then were subjected to 2 treatments: (T1) 80 µg of Jaburetox and (T2) sodium phosphate dialyzing buffer. The experimental design was completely randomized with 3 repetitions. The experimental unit was 10 Petri dishes (5.0 cm × 0.8 cm, lined with wet filter paper) containing 1 larva each.

Through d 4 (5 doses per d 0–4), the larvae were supplied daily with 1 cm^2 leaf discs coated with 16 µg of Jaburetox peptide diluted in 7.3 µL of sodium phosphate buffer and 0.01% of Silwet® (Surfactant), totaling 80 µg peptide in 5 cm^2 leaf disc offered to each larva. For T2, 7.3 µL of sodium phosphate with surfactant was added on the leaf discs. The solutions were applied on top of the discs, which were dried at room temperature before being offered to the larvae. From d 5 on, uncoated leaf discs were renewed every 48 h, and the experiment was monitored until d 9.

EVALUATIONS AND DATA ANALYSIS

Larvae mortality was evaluated every d and changes in instar were observed by retrieving the larval cephalic capsules. Leaf area consumption (cm) was evaluated every 48 h (at the time of leaf disc renewal) by calculating the difference between the initial and the remaining area. The final larval weight was evaluated at the end of the neonate larvae experiment. In the third instar feeding experiment, the individual weight of live larvae was evaluated at d 0, 3, 6, and 9.

After confirming normal distribution using the homogeneity of variance test, the neonate mortalities were subjected to analysis of variance and the averages compared using the Tukey test, with significance limit set at 5% probability of error. An independent ANOVA was applied to each d analyzed. The percentage data were transformed by Arcsine square root (× + 0.5)/100, and the numbers transformed by the Log (× + 1). An unpaired Student *t*-test was applied to the bioassay third instar data and $P < 0.05$ was considered significant.

Results

TOXICITY OF THE TWO VERSIONS OF THE JABURETOX PEPTIDE ON NEONATAL *HELICOVERPA ARMIGERA* LARVAE

Mortality was significantly higher in neonates fed with peptides versus controls throughout the experiment. The Jaburetox Δ-β peptide induced about 40% mortality (about 30% higher than controls by 2 d of feeding, while both versions of the Jaburetox peptides caused increased mortality to about 60% by d 4 (Fig. 1a). After toxin was withdrawn (on d 4), mortality continued to increase, reaching about 75% by the termination of the assay at 8 d from the beginning of the experiment (Fig. 1a). On the contrary, control larval mortality remained below 10% for the duration of the experiment (8 d) (Fig. 1a; $P = 0.0001$; see Table 1). No statistical difference in mortality was observed between the 2 controls used, indicating that the sodium phosphate buffer (20 mM pH 7.5) and the surfactant, in which the peptides were diluted, did not cause significant mortality among the insects.

During the first 4 d of feeding, mortality was higher when neonates were fed the Jaburetox Δ-β peptide (65.4% at d 4) in comparison to the full-length Jaburetox (49% at d 4) ($P = 0.0001$), but at d 6 and 8, both peptides were equally efficient in inducing mortality (Fig. 1a).

Both peptides did influence feeding behavior. The control larvae continually increased their consumption from 20% to over 90% over the entire time of the experiment (Fig. 1b). The leaf consumption by the larvae fed with either Jaburetox peptides remained significantly lower than the control from d 4 to the end of the experiment (Fig. 1b). At d 8, individual surviving larvae consumed 20% of the Jaburetox coated leaf disc, while the ones fed on Jaburetox Δ-β peptide consumed 47.4%, and the control larvae consumed about 94% of the offered tissue (1 cm^2) (Fig. 1b) ($P = 0.0050$). Among live larvae on d 8 of the experiment, the larvae fed with the full length Jaburetox consumed 79% less than the control, and 58% less than the larvae fed with the Jaburetox Δ-β peptide. Because of the apparent longer-lasting effect of Jaburetox on feeding behavior, this peptide was selected to be used in subsequent assays.

Although Jaburetox caused a significant reduction in leaf consumption on a per larva basis, there was no significant difference in weight of live treated and non-treated larvae by d 8 (data not shown). Despite no significant difference in weight between treated and control larvae, the larvae that survived until d 8 after being fed with either peptide (around 30%) presented delayed development in comparison with both the controls, with none reaching the fifth instar (Fig. 2).

TOXICITY OF THE FULL-LENGTH JABURETOX VERSION TO THIRD INSTAR *HELICOVERPA ARMIGERA* LARVAE

The effect of full-length Jaburetox on *H. armigera* also was evaluated in older larvae that were allowed to develop to the third instar stage on artificial diet prior to initiating the trial. Individual third instar larvae were removed from the diet and fed with 16 µg Jaburetox treated or control leaves applied daily for 5 consecutive d (from d 0 to 4), and for d 6 through 9 they were fed untreated leaves. The experiment was monitored until d 9. The accumulated mortality increased over time. By 9 d from the beginning of the feeding assay, Jaburetox treatment caused 100% mortality, whereas in the controls (phosphate buffer) the accumulated mortality was only 30% (Fig. 3a) ($P = 0.0003$). Also, mortality continued to rise from about 36% to 100% after toxin feeding was stopped at d 5.

Feeding third instar larvae with Jaburetox did not affect their consumption behavior, because no difference was observed in the consumption between remaining live larvae treated with Jaburetox and

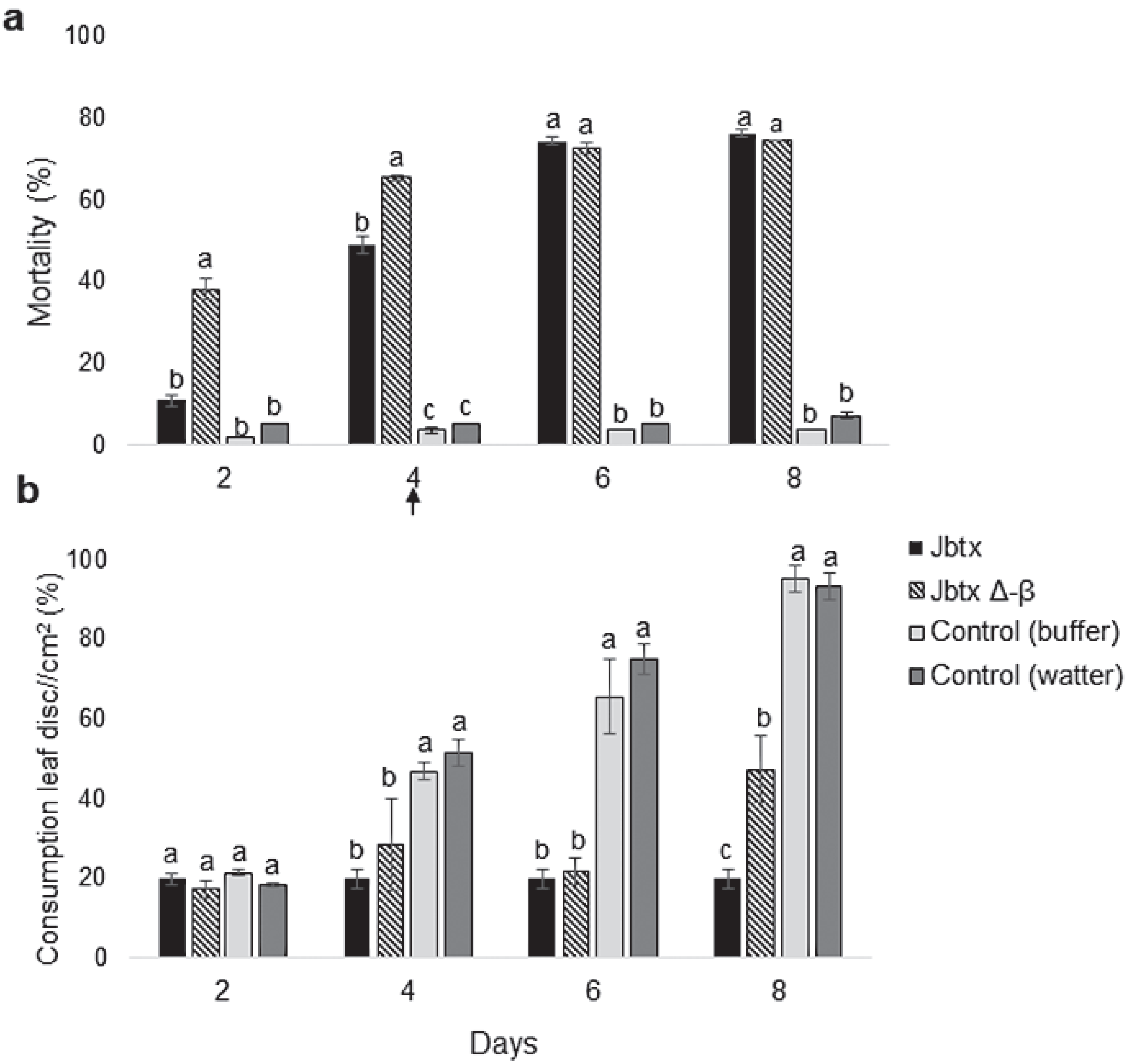

Fig. 1. Feeding assay with neonate *Helicoverpa armigera* larvae on maize leaf discs coated with Jaburetox (Jbtx) and Jaburetox Δ-β (Jbtx Δ-β). (a) Accumulated mortality; (b) daily consumption. The arrow indicates the d Jaburetox toxin feeding was stopped. Means with the same letter within the same d do not differ with each other (Tukey test, $P < 0.05$).

the controls through the first 8 d of the experiment (Fig. 3b; Table 1). In the last d of the experiment (d 9 after the first dose of toxin was offered), the control larvae ate almost 82% of the supplied leaf tissue and the treated larvae ate only 14%, but during this time all treated larvae remaining after d 8 died. The total amount of peptide consumed by the third instar larvae was approximately 36 µg of the 80 µg of peptide supplied to them (around 45% of coated leaf consumed, based on the consumption until d 5). This amount was enough to cause 100% mortality in 9 d (Fig. 3a).

There was a significant effect of Jaburetox feeding on weight of the remaining live larvae at d 3, where the peptide caused an increase of 25% in weight compared with the control (Fig. 3c; Table 1). These results may be explained by the water retention effect of the Jaburetox due to diuresis inhibition, as demonstrated by Stanisçuaski et al. (2009). However, at d 6, no difference in weight was observed between remaining live treated and non-treated larvae (93 mg on average) (Fig. 3c). The control larvae gained from 98 to 230 mg in weight from d 6 to d 9.

The entomotoxic peptide Jaburetox also caused a delay in development. Only 10% of the Jaburetox fed larvae reached the fourth instar and none reached the fifth instar stage by the end of the experiment. With control larvae, there was a continuous developmental process that led to the majority (86%) of the larvae reaching the fifth instar by d 9 (Fig. 4a). Figure 4b shows a representative comparison between Jaburetox treated larvae and control larvae at d 8.

Table 1. Statistical support data: *F*, *t* and *P* values for specified experiments.

Experiment	Variable	Day	*F* value	*P*
Neonate feeding (df 3, 12)	Accumulated mortality	2	11.27	0.008
		4	5.68	0.0117
		6	17.90	0.001
		8	19.88	0.0001
	Daily consumption	2	1.43	ns
		4	6.40	0.007
		6	53.84	0.000
		8	7.22	0.0050
Experiment	**Variable**	**Day**	***t* value**	***P***
Third instar feeding (df 1, 8)	Accumulated mortality	3	0.0	1.000
		4	0.78	0.456
		5	1.77	0.114
		6	6.77	0.0001
		7	6.70	0.0001
		8	5.40	0.0006
		9	5.90	0.0003
	Daily consumption	2	1.73	0.121
		3	0.20	0.841
		4	1.79	0.109
		5	1.19	0.265
		6	0.98	0.355
		7	1.11	0.295
		8	0.95	0.391
		9	6.22	0.0002
	Weight	3	2.96	0.0180
		6	1.07	0.314

In total, these results demonstrated that both forms of Jaburetox, the full version Jaburetox, and Jaburetox Δ-β, when offered orally are equally toxic to *H. armigera* neonate larvae inducing about 75% mortality by d 8 of feeding. If feeding is initiated with third instar larvae, the full version of Jaburetox kills 100% of the larvae by d 9 of feeding.

Discussion

Our results demonstrated for the first time that the Jaburetox peptide is toxic to the multi-host insect-pest *H. armigera*, with a high level of mortality achieved in different larval instars. When the 2 peptide versions were supplied to neonate larvae, the Jaburetox Δ-β induced 25% more larval mortality compared with the full version Jaburetox after 4 d of feeding. However, by 6 d, the mortality for both peptides was 75% in comparison to controls and remained at this level through the final 8 d evaluation even though peptide feeding was stopped at d 4.

The quick response in mortality observed in larvae fed with the short version of Jaburetox (Jaburetox Δ-β) may be due to its smaller size (79 amino acids) compared to the full version Jaburetox (93 aa). The Jaburetox Δ-β may have been absorbed more easily by the larval digestive system, providing a more rapid entomotoxic effect. The smaller size of this peptide is due to the removal of a prominent region, called the β-hairpin, which previously was considered an important motif that could be partly responsible for the toxicity to insects (Martinelli et al. 2014). It was verified subsequently that the entomotoxic domain is found in the N-terminal portion of Jaburetox, and the

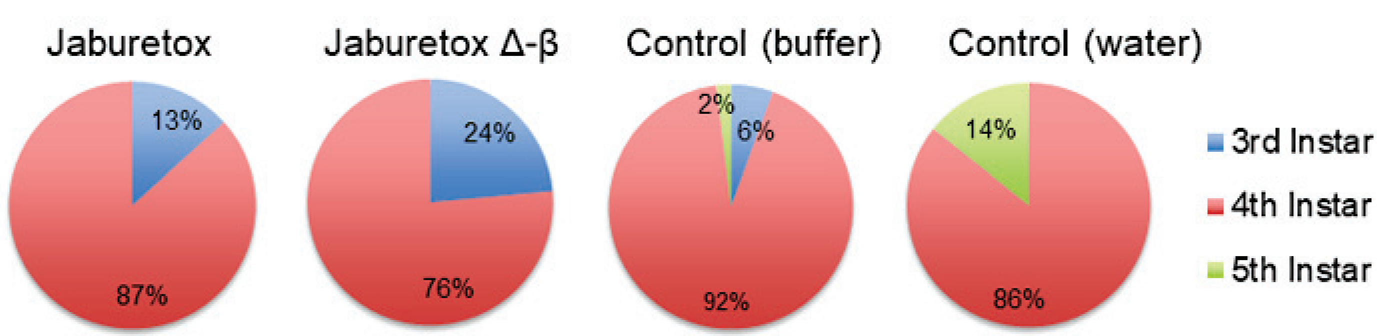

Fig. 2. Percentage of neonate larvae fed with leaf discs treated with Jaburetox, Jaburetox Δ-β, or control solutions that reached the third, fourth, and fifth instar at d 8 of experiment.

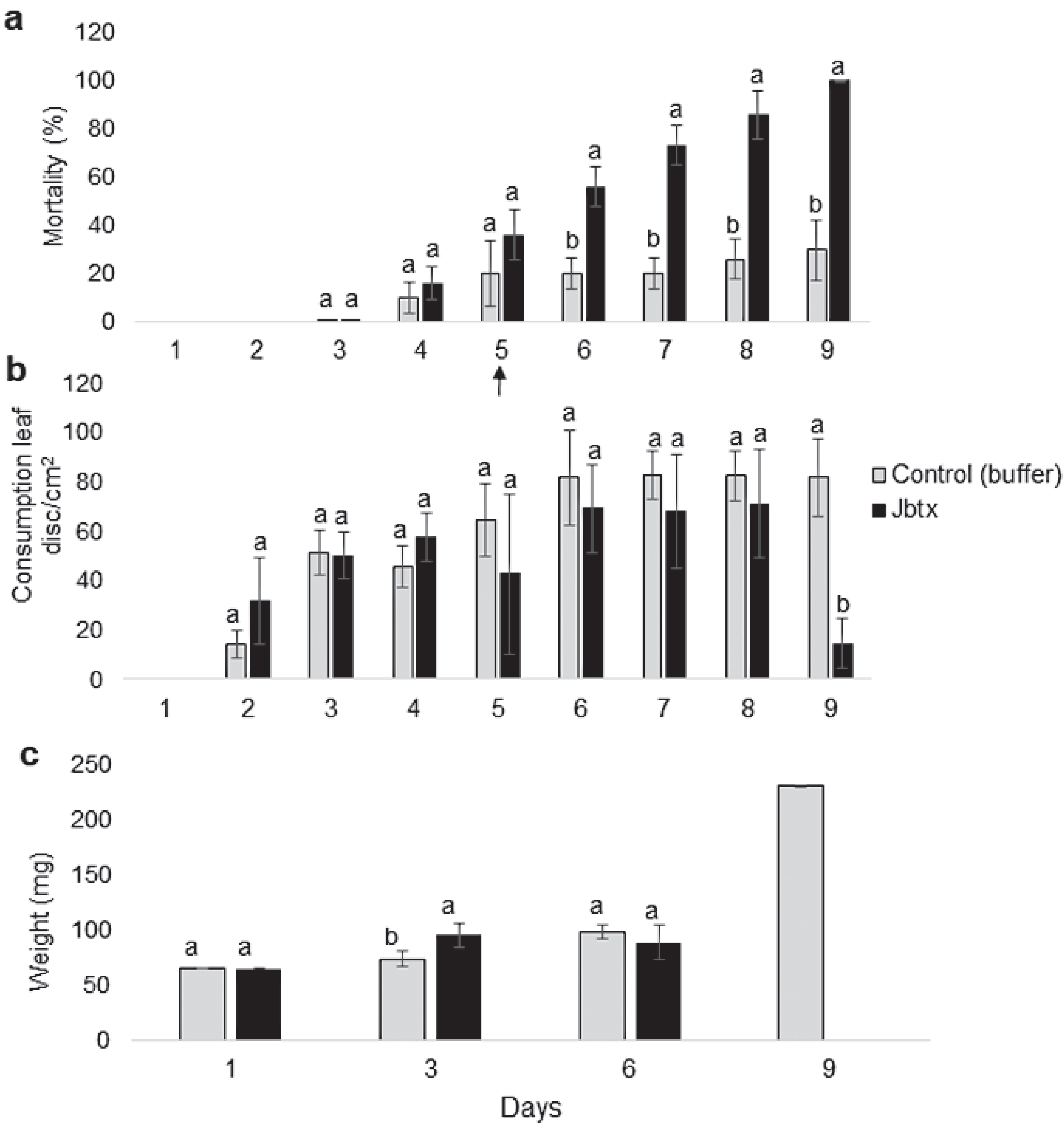

Fig 3. Feeding assay with third instar *Helicoverpa armigera* larvae on maize leaf discs coated with Jaburetox (Jbtx). (a) Accumulated mortality; (b) daily consumption; (c) larvae weight. The arrow indicates the d Jaburetox toxin feeding was stopped. Means with the same letter within the same d do not differ from each other (Student *t*-test, P < 0.05).

peptide without the β-hairpin region (C-terminal) caused mortality in *R. prolixus* injected nymphs equivalent to that of the original Jaburetox (Martinelli et al. 2014). However, when offered orally to *R. prolixus*, this similarity was not so evident in terms of mortality.

Our data agrees with the above model, because we show that the 2 forms of entomotoxic peptide are effective equally in killing neonate larvae, confirming that the region of β-hairpin is not necessary to cause mortality when introduced orally to *H. armigera*. In fact, the absence of the Δ-β causes quicker mortality in neonates. However, the full-length Jaburetox appears to have longer lasting effects after cessation of feeding suggesting the β-hairpin adds biological stability to the peptide within the insect. The lower leaf consumption by the full-length Jaburetox fed neonate larvae supports this idea.

The full version of Jaburetox induced 76% mortality and almost 80% reduction in consumption when it was offered orally to neonatal larvae on maize leaf discs. In older larvae (third instar), the treatment

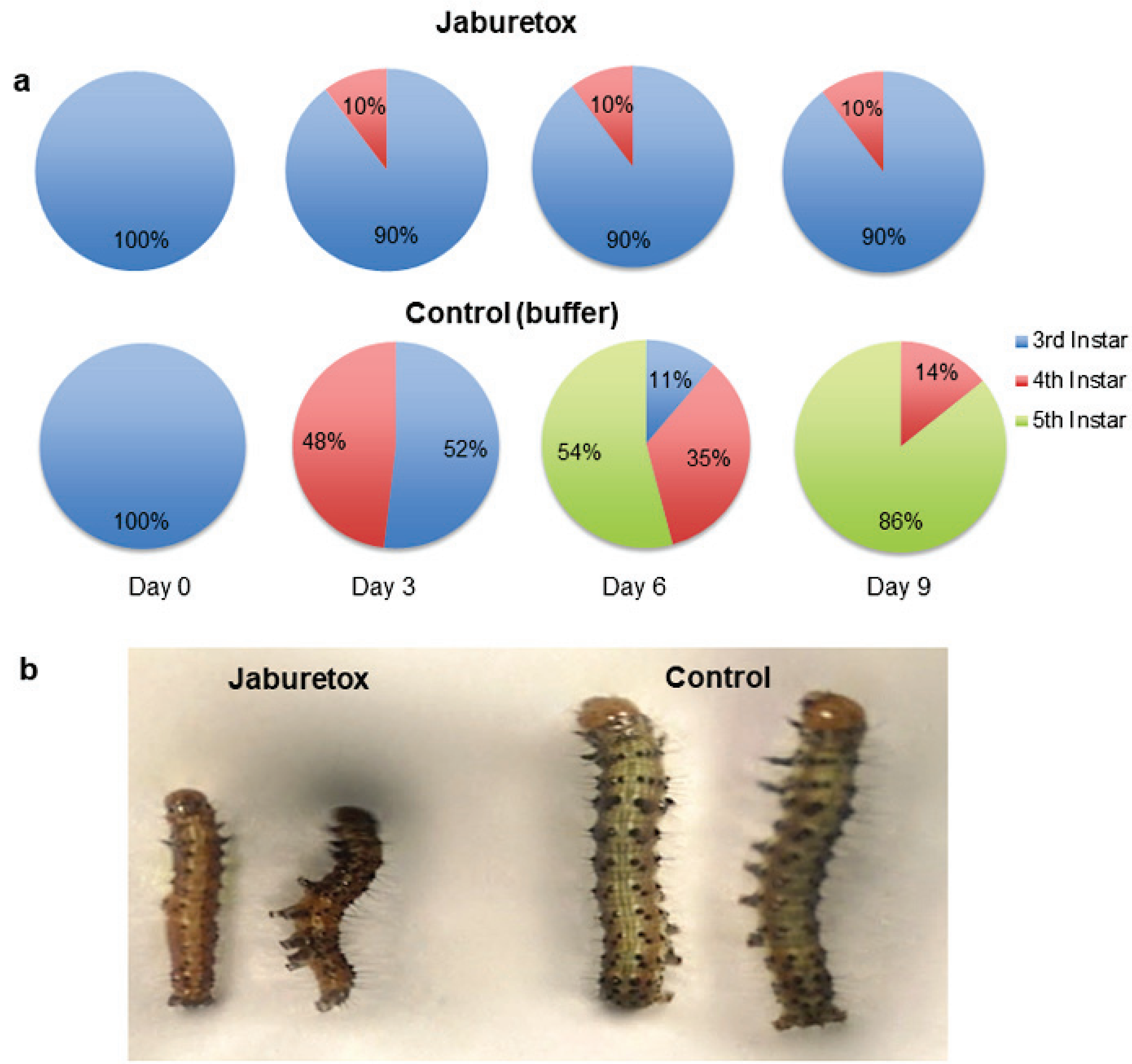

Fig. 4. (a) Percentage of third instar larvae fed with leaf discs treated with Jaburetox and control solution that reached the third, fourth, and fifth instar along the experiment; (b) larvae observed on d 8 of bioassay (1 d before all Jaburetox treated had died): on the left, Jaburetox larvae are in third instar (80 µg per 5 cm^2); on the right, control larvae are in the fifth instar.

with Jaburetox resulted in 100% mortality in experiments where the control induced only 30% mortality. These mortality rates resulted from neonatal larvae ingesting a total of 6.5 µg of Jaburetox, and third instar larvae ingesting a total of 36 µg of Jaburetox per insect. This calculation was based on the total amount of protein offered and consumed by each larvae. Similar to our findings, Mulinari et al. (2007) obtained 100% mortality after 8 d of feeding third instar *S. frugiperda* larvae with 50 µg cm^2 of Jaburetox. Therefore, this peptide has activity against multiple members of the Lepidoptera. Although the final mortality in our study was less for the neonate experiment than for the third instar larvae experiment, the Jaburetox entomotoxic peptide initially killed the younger neonates and larvae quicker (a significance of about 45% mortality after 4 d of feeding initiated with neonates and less than 20% mortality that was not significantly different than controls at the same timepoint for feeding initiated with third instar larvae). The experiment was not given more time because the maize leaf discs as a source of feeding does not furnish enough nutrition for the larva to complete its biological cycle. In natural conditions in the field, the larvae migrate to the ear to consume the grain to complete its cycle.

The dynamics of leaf consumption by the older larvae (third instar) were quite different from the neonates. Neonate larvae fed with full version Jaburetox showed approximately 80% reduction in consumption compared with the controls; however, third instar larvae fed with the same peptide did not show any reduction in consumption, consuming as much as the controls until all died by d 8.

In our experiment with the third instar larvae, the Jaburetox feeding resulted in an early larvae weight increase higher than the control larvae (by d 3), suggesting that the ingested food may be kept in the digestive system, not being absorbed and used as a nutritional source and, as a result, the insects are stimulated to continue to feed in an attempt to gain required nutrition. Also, the temporary insect weigh gain could be due the diuresis inhibition effect of Jaburetox that would cause an inability to remove water. Based on previously published information on modes of action of these peptides (see below), it is likely that our result is due to a combined effect on digestive tract membrane integrity and diuresis.

Jaburetox's insecticidal action mechanism has not been completely explained, but it is shown to have several biological activities including alteration of cellular membrane integrity, as well as fungicidal and bactericidal activity (Becker-Ritt et al. 2017). It is known that, in the hemipteran disease vector, *R. prolixus*, diuresis inhibition and probable electrolytic imbalance occurs, with alterations in the levels of cyclic GMP (Guanosine 3´, 5´-cyclic monophosphate) and in the transmembrane potential of the Malpighian tubules (Stanisçuaski et al. 2009).

Studies with artificial systems also have shown that Jaburetox is capable of interacting with lipid bilayers, affecting liposome permeability (Barros et al. 2009; Martinelli et al. 2014) and forming ionic channels (Piovesan et al. 2014), but without causing lysis of the particles or cells. The ability of Jaburetox to insert itself into the lipid layer of liposomes, and thus alter physical properties of the membrane (Micheletto et al. 2016) is probably the physical-chemical basis for its multiple biological effects, including toxicity to insects.

In another study, after injecting Jaburetox into the hemipteran *T. infestans*, the insects presented paralysis in their legs and uncoordinated antenna movements, suggesting neurotoxic effects which preceded death. The peptide was located in the insects' central nervous system, in which significant reductions were observed in the content of the neurotransmitter nitric oxide and in the activity of the enzyme responsible for its formation, nitric oxide synthase (Galvani et al. 2015). It was postulated that Jaburetox inhibits the enzyme activity of the nitric oxide synthase (NOS), responsible for the production of the neurotransmitter nitric oxide, and also modulates UDP-N-Acetylglucosamine pyrophosphorylase, a key enzyme in chitin synthesis and glycosylation pathways (Galvani et al. 2015; Fruttero et al. 2017). Chitin in insects has an important role as a component of the cuticle, salivary glands, trachea, and peritrophic matrix (Merzendorfer 2011). Galvani et al. (2015) established that UDP-N-Acetylglucosamine pyrophosphorylase physically interacted with Jaburetox in the central nerve system causing an elevation of the UDP-N-Acetylglucosamine pyrophosphorylase enzyme activity. Fruttero et al. (2017) observed that Jaburetox triggers a decrease in the expression of mRNA of UDP-N-Acetylglucosamine pyrophosphorylase and chitin synthetase.

Jaburetox also affects the immune system of *R. prolixus*, causing aggregation of hemocytes and morphological alterations, suggesting apoptosis in these cells, and thus compromising the insects' response to challenges from entomopathogenic bacteria (Fruttero et al. 2016). It has been reported that the peptide is lethal to the insect plant pest *S. frugiperda* (Mulinari et al. 2007), but no studies have been done to investigate the mechanism of action in Lepidoptera. Our results showing increased mortality demonstrate the potential for the peptides to have a similar effect on a broad array of Lepidoptera.

Importantly, all results to date indicate that the Jaburetox peptide is innocuous to mammalian species. In biosafety analyses, Jaburetox was administered to adult and neonatal rats via intraperitoneal injection and orally via an intragastric tube. In these experiments, there was no observable toxicity (Mulinari et al. 2007). In addition, Sa et al. (2020) performed the risk assessment of the peptide Jaburetox following the general recommendations proposed by the International Life Sciences Institute (Delaney et al. 2008). The amino acid sequence of Jaburetox showed no relevant similarity to allergenic, antinutritional, or toxic proteins, and no hazards for Jaburetox were reported from the history of use. Thus, the Jaburetox offers potential for use in transgenic insect resistant crop strategies.

The absence of potential risks of Jaburetox together with the results from the present experiments support further evaluation of the use of the Jaburetox peptide in transgenic crops for insect resistance. The continuous expression of Jaburetox in genetically modified plants may represent an excellent strategy for controlling *H. armigera* in an environmentally sustainable strategy to reduce the economic impact of this worldwide pest. As a continuation of this work, we produced transgenic maize plants expressing the Jaburetox gene that are under evaluation for *H. armigera* and *S. frugiperda* control.

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