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Imidacloprid impairs the post-embryonic development of the midgut in the yellow fever mosquito *Stegomyia aegypti* (=*Aedes aegypti*)

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Abstract. The mosquito *Stegomyia aegypti* (=*Aedes aegypti*) (Diptera: Culicidae) is a vector for the dengue and yellow fever viruses. As blood digestion occurs in the midgut, this organ constitutes the route of entry of many pathogens. The effects of the insecticide imidacloprid on the survival of *St. aegypti* were investigated and the sub-lethal effects of the insecticide on midgut development were determined. Third instar larvae were exposed to different concentrations of imidacloprid (0.15, 1.5, 3.0, 6.0 and 15.0 p.p.m.) and survival was monitored every 24 h for 10 days. Midguts from imidacloprid-treated insects at different stages of development were dissected and processed for analyses by transmission electron microscopy, immunofluorescence microscopy and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assays. Imidacloprid concentrations of 3.0 and 15.0 p.p.m. were found to affect midgut development similarly. Digestive cells of the fourth instar larvae (L4) midgut exposed to imidacloprid had more multilamellar bodies, abundantly found in the cell apex, and more electron-lucent vacuoles in the basal region compared with those from untreated insects. Moreover, imidacloprid interfered with the differentiation of regenerative cells, dramatically reducing the number of digestive and endocrine cells and leading to malformation of the midgut epithelium in adults. The data demonstrate that imidacloprid can reduce the survival of mosquitoes and thus indicate its potentially high efficacy in the control of *St. aegypti* populations.

Key words. Cell proliferation, digestive cells, mosquito control, neonicotinoid, sub-lethal effects.

Introduction

The mosquito *Stegomyia aegypti* (=*Aedes aegypti*) (Linnaeus, 1762) is a major vector of the yellow fever and dengue viruses, which cause morbidity and mortality in humans. The combat of these diseases involves control of the vector with insecticides (Pridgeon *et al.*, 2008; Coelho *et al.*, 2009; Allan, 2011). Currently, neurotoxic organophosphate insecticides and pyrethroids are commonly used to control *St. aegypti*. However, the careless use of these insecticides in the last 30 years has led to increased levels of resistance in mosquito populations in parts of Asia and Latin America (Rodríguez *et al.*, 2007; Martins *et al.*, 2009; Lima *et al.*, 2011), necessitating the identification of new insecticides to control mosquitoes.

The neonicotinoid imidacloprid has a broad spectrum of activity, persistence and versatility of application in agriculture and is one of the most commonly used pesticides worldwide (Elbert *et al.*, 2008; Jeschke & Nauen, 2008). However, little information is available about its effects on *St. aegypti* (Antonio-Arreola *et al.*, 2011). Although imidacloprid has low activity against

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larvae and pupae compared with other insecticides, its sub-lethal effects may be important in reducing populations of *St. aegypti* (Tomé *et al.*, 2014).

The midgut of *St. aegypti* has been an important focus of studies seeking to block the transmission of pathogens as the midgut is the location of blood digestion and can be considered the 'gateway' of entry for various pathogens, including viruses (Chauhan *et al*., 2012 Wang *et al*., 2012; Kading *et al*., 2014). The midgut consists of a simple epithelium surrounded by two muscle layers. There are three cell types in the midgut epithelium of *St. aegypti*, comprising, respectively, digestive, regenerative and endocrine cells. The digestive cells, which contain microvilli, are responsible for the production of digestive enzymes and absorption of nutrients. The regenerative cells (also called stem cells) are undifferentiated and are responsible for the renewal of cells within the midgut epithelium. The endocrine cells in this tissue are thought to regulate the functions of the epithelium by mediating the secretion of substances into the haemolymph (Hecker *et al.*, 1971; Hecker, 1977; Brown *et al.*, 1985; Glattli *et al.*, 1987; Moffett & Moffett, 2005).

During metamorphosis, the larval digestive cells of *St. aegypti* degenerate and are replaced through the differentiation and proliferation of regenerative cells (Nishiura *et al.*, 2003; Ray *et al.*, 2007; Fernandes *et al.*, 2014). Renewal of the midgut occurs at different times in the anterior and posterior regions, suggesting that this process is quite complex (Fernandes *et al.*, 2014). Changes in the course of midgut restructuring during metamorphosis can be harmful to the mosquito. Therefore, interference with post-embryonic development of the midgut may have the potential to negatively impact upon the process of feeding and thereby reduce fecundity.

In the present study, the effects of the insecticide imidacloprid on survival and post-embryonic development of the midgut of *St. aegypti* were investigated.

Materials and methods

Mosquitoes

Larvae of *St. aegypti* (PPCampos strain, Campos dos Goytacazes) were obtained from a colony maintained in the insectary of the Department of General Biology, State University of Viçosa (UFV), Viçosa, MG, Brazil. The larvae were raised in plastic trays with distilled water and were provided with turtle food (Reptolife; Alcon Pet Ltda, Camburiú, SC, Brazil). Insects were maintained under conditions of controlled temperature (25 ± 5 °C), relative humidity (60 ± 5%) and a defined photoperiod (LD 12 : 12 h).

A total of 170 larvae [fourth instar (L4)], 510 female pupae in different developmental phases [white pupae (WP) collected immediately after ecdysis and pupae collected at 24 h and 48 h (P24 and P48, respectively) after ecdysis], and 170 newly emerged (NE) adult females were used in the morphological analyses. Female pupae were separated from males according to size. Females (except L4) were selected in order to standardize the sample and avoid the effects of possible differences between male and female development times. In addition, only females are considered important as vectors of pathogens.

Survival

Imidacloprid [Evidence® 700 WG, 700 g active ingredient (a.i.)/L, water-dispersible granule; Bayer CropScience Ltda, São Paulo, SP, Brazil] was diluted in distilled water. A total of 600 larvae [third instar larvae (L3)] were separated into four batches (replicates) of 25 insects per batch for each of five concentrations (0.15, 1.5, 3.0, 6.0 and 15.0 p.p.m., respectively) of insecticide and a control treatment without insecticide (i.e. only water) for the survival analysis. Insects were placed in 500-mL plastic containers filled with 200 mL distilled water with or without imidacloprid at the respective concentrations and 10 mg turtle food (Tomé *et al.*, 2014).

Insect survival was assessed every hour for the first 12 h and then daily for 10 days, which was sufficient for the insects to reach the adult stage. Insects were considered dead if they were unable to move when prodded with a brush of fine hair and were then removed from the test containers. Survival was calculated by dividing the number of dead insects by the initial number of insects (Tomé *et al.*, 2014).

Insects from the 3.0 and 15.0 p.p.m. treatments were used in morphology, transmission electron microscopy (TEM), cell damage and immunofluorescence assays because these insects showed lower rates of survival compared with the controls. We selected the 3.0 and 15.0 p.p.m. treatments because the former promoted an intermediate effect (~40% mortality) and the latter induced higher disorder (~80% mortality).

Morphology

The midguts of insects from each treatment (control, 3.0 and 15.0 p.p.m. imidacloprid) were dissected in saline solution (0.1 M NaCl, 20 mM KH_2PO_4, 20 mM Na_2HPO_4) and fixed in Zamboni's fixative (paraformaldehyde plus picric acid) for 2 h. After fixation, 20 midguts from each stage of mosquito were separated, washed three times with distilled water, stained with diamidino-2-phenylindole (DAPI; Biotium, Inc., Hayward, CA, U.S.A.) for 30 min, and washed again in distilled water. Midguts were then mounted with Mowiol anti-fading solution (Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil) and photographed using an optical Olympus BX60 microscope coupled with a digital Olympus Q-Color3 camera (Olympus Corp., Tokyo, Japan).

To separate and count digestive and regenerative cells, six areas of the anterior and posterior midgut were randomly selected and photographed with a 40× objective (total area: 0.414 mm^2) as previously described (Fernandes *et al.*, 2014). Cell counts were determined, and cells were classified as digestive cells (cells with larger nuclei present in the apical region of the digestive epithelium) or regenerative cells (cells with small nuclei present in the basal region of the digestive epithelium), as described by Ray *et al.* (2007); digestive cells were further classified as either completely or not completely differentiated. All measurements and cell counts were made using Image-Pro Plus Version 4.5 (Media Cybernetics, Inc., Silver Spring, MD, U.S.A.).

Immunofluorescence

Observations of proliferating cells were validated using antibodies targeting the nuclear protein phospho-histone H3 *in situ* (Preuss *et al.*, 2003). The midguts (10 from each stage/phase) of each treatment (control, 3.0 and 15.0 p.p.m. imidacloprid) were previously fixed and washed (see Morphology) and incubated for 24 h at 4 °C in anti-phospho-histone H3 primary antibody solution (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.), diluted 1 : 100 in phosphate-buffered saline (PBS) with 1% Triton X-100 (PBST). After this step, the samples were washed three times with PBS and incubated for 24 h at 4 °C with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma-Aldrich Brasil Ltda) diluted 1 : 500 in PBS, and then submitted to three 10-min washes with PBS. Phospho-histone H3-positive cells show a bright green nucleus under ultraviolet light.

Fixed midguts (10 from each stage/phase) were washed in PBST and then incubated for 24 h at 4 °C in anti-FMRFamide primary antibody solution (Peninsula Laboratories, Inc., San Carlos, CA, U.S.A.), diluted 1 : 400 with 1% PBST. Thereafter, the samples were washed with PBS three times (5 min each) and incubated for 24 h at 4 °C with FITC-conjugated secondary antibodies (Sigma-Aldrich Corp., St Louis, MO, U.S.A.) diluted 1 : 500 in PBS, and then submitted to three washes with PBS. After staining, FMRFamide-positive cells appear as bright green cells.

As a positive control, six midguts from insects at the L4 stage were used as phospho-histone H3-positive cells (Fernandes *et al.*, 2014). As a negative control, five midguts from each mosquito developmental stage were used and treated as described above, excluding the primary antibody incubation.

The slides were mounted in Mowiol, analysed and photographed using an epifluorescence microscope. Endocrine cells and dividing cells throughout the whole midgut were counted. We expected to find increased numbers of proliferating cells (i.e. cells exhibiting bright green fluorescence) in control insects compared with insects exposed to imidacloprid. The absence of brightness would demonstrate the inability of cells to undergo normal proliferation.

Transmission electron microscopy

Transmission electron microscopy was used to evaluate cell injury in the midgut induced by imidacloprid in three individuals at each developmental stage for each treatment (control, 3.0 and 15.0 p.p.m. imidacloprid). Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) for 2 h. After fixation, the midgut fragments were washed in cacodylate buffer and post-fixed in 1% osmium tetroxide for 2 h in the dark. Following post-fixation, the specimens were washed twice in 0.1 M PBS, dehydrated in a series of increasing ethanol concentrations (70–100%), and pre-infiltrated in LR White resin (London Resin Company Ltd, Berkshire, England) solution and 100% ethanol (2 : 1) for 1 h. Subsequently, the samples were embedded in pure resin and maintained at room temperature for 16 h, followed by polymerization in gelatine capsules (Electron Microscopy Sciences, Inc., Hatfield, PA, U.S.A.) at 60 °C for 24 h.

Ultra-thin sections were placed on copper grids and incubated for 20 min in 1% aqueous uranyl acetate and lead citrate. The samples were observed and photographed using a scanning electron microscope (SEM) (Zeiss EM 109; Carl Zeiss GmbH, Jena, Germany) at the Núcleo de Microscopia e Microanálise (NMM) at UFV.

DNA fragmentation

The midguts of *St. aegypti* begin remodelling in the late larval stage with the death of the digestive cells; this process continues for up to18 h after pupation (Nishiura & Smouse, 2002; Nishiura *et al.*, 2003). Therefore, to study the effects of imidacloprid in the cell genome, we examined DNA fragmentation as indicative of cell death in P24 and P48, phases in which the regenerative cells are differentiating (Ray *et al.*, 2007; Fernandes *et al.*, 2014).

Five midguts of P24 and P48 for each treatment (control treatment, 3.0 p.p.m. imidacloprid treatment and 15.0 p.p.m. imidacloprid treatment) were fixed and washed (see Morphology). Posteriorly, the samples were dehydrated in increasing concentrations of ethanol (70–100%) and embedded overnight in Leica Historesin (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). The samples were subsequently embedded in historesin with hardener and subjected to microtomy. Three sections of each midgut (3 μm thick) were transferred to glass slides and treated with proteinase K (10 μM/mL of 10 mM Tris-HCl, pH 7.4) for 1 h at 37 °C. Cell damage was labelled with an *In Situ* Cell Death Detection Fluorescein kit (Roche Applied Science, Penzberg, Germany) using terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), during which the enzyme catalyses a reaction between fragmented DNA (3′-OH) and a nucleotide associated with a fluorophore (FITC). The slides were washed three times in 0.1 M phosphate buffer (pH 7.4) and incubated in reaction buffer for 45 min at 37 °C, according to the manufacturer's instructions. The slides were washed, covered with Mowiol and photographed as described above (see Morphology). TUNEL-positive cells (i.e. cells with fragmented DNA) exhibited green fluorescent nuclei. We analysed the presence or absence of cell death under the assumption that pupae not exposed to pesticides will not exhibit cell death during this developmental period (Nishiura & Smouse, 2002; Nishiura *et al.*, 2003; Ray *et al.*, 2007).

As a negative control for the TUNEL reaction, one histological section of the midgut from insects of each phase was incubated without transferase enzyme, according to the manufacturer's protocol.

Statistical analysis

The results of the survival assay were analysed using the non-parametric LIFETEST (SAS Institute, Inc., 2008), for which survival curves were obtained using Kaplan–Meyer estimates. Insects that emerged were classified as censored data. Survival

curves were compared using Holm–Sidak tests. Cell counts were subjected to one-way analysis of variance (ANOVA) for normally distributed variables and Kruskal–Wallis tests for variables without normal distributions.

Results

Survival

Survival (time–mortality response) differed significantly among insects treated with different concentrations of insecticide (log-rank test, $\chi^2 = 183.49$, d.f. = 5, $P < 0.001$). A decrease in concentration-dependent survival was observed after 10 days of treatment, and the highest concentration of 15.0 p.p.m. caused death in about 80% of insects at the endpoint. The effects of the 0.15 p.p.m. concentration were similar to those of the control, and the effects of the 1.5 p.p.m. concentration were similar to those of the 3.0 p.p.m. concentration in terms of survival (Holm–Sidak test, $P > 0.05$).

Morphology

The morphology of the *St. aegypti* midgut was affected by treatment with imidacloprid at both 3.0 and 15.0 p.p.m. Compared with the control, imidacloprid treatment caused disruptions in digestive and regenerative cells, including deformation of the nucleus and large spaces between digestive cells; imidacloprid concentrations of 3.0 and 15.0 p.p.m. caused similar morphological changes; no dose-dependent response was observed (Fig. 1).

Numbers of digestive cells were similar in treated and untreated WP. However, in P24, the number of digestive cells in control insects increased until the end of development (NE), but remained low in treated individuals ($P < 0.05$) (Fig. 2A).

In the control treatment, the number of regenerative cells was greater at L4 and decreased at the P24 stage. By contrast, in treated insects at the L4 stage, the number of regenerative cells was reduced, but increased during development, resulting in greater numbers of regenerative cells in later stages compared with the control ($P < 0.05$) (Fig. 2B).

Immunofluorescence

Cell proliferation was analysed by assessing the level of histone H3 phosphorylation following imidacloprid treatment. Interestingly, positive phospho-histone H3 staining was observed in all developmental stages of *St. aegypti*, and the number of positively stained cells did not change in the midguts of control insects. However, in imidacloprid-exposed insects, phosphorylation of histone H3 was reduced in comparison with the control ($P < 0.05$) (Figs 2C and 3).

Endocrine cells were found in the posterior region of the midgut of L4 control insects (Figure S1, online), but in the posterior and anterior–mid regions of insects treated with imidacloprid. Surprisingly, fewer FMRF-positive cells were observed in L4 control insects than in L4 imidacloprid-treated insects. However, from P24, the number of cells increased in the control, including NE, although this effect was not observed in imidacloprid-treated insects ($P < 0.05$) (Fig. 2D and Figure S1).

Transmission electron microscopy

In individuals treated with imidacloprid, digestive cells had more multilamellar bodies, found abundantly in the cell apex, and more electron-lucent vacuoles in the basal region compared with those in the control (Figs 4A, B and 5A, B). Digestive cells derived from the L4 stage were in the process of degeneration in both treated and control insects at the WP stage. However, in the control, the cytoplasm of digestive cells was electron-lucent, with various multilamellar bodies, whereas that of digestive cells in treated WP insects contained electron-lucent large vesicles in the basal region and disrupted microvilli (Figs 4B and 5B).

In the midgut epithelium of P24 control individuals, digestive cells were in the process of differentiation and cell debris from L4 digestive cells was observed (Fig. 4C). However, in P24 imidacloprid-treated individuals, the cytoplasm of regenerative cells was almost completely occupied by large vacuoles, and the plasma membranes in some differentiating cells were disrupted and cytoplasmic contents released in the lumen of the midgut (Fig. 5C). After exposure to imidacloprid, digestive cells in individuals at the P48 (Fig. 5D) and NE (not shown) stages also possessed vacuolated cytoplasms with multilamellar bodies, and some cells exhibited broken membranes.

DNA fragmentation

TUNEL assays revealed fragmented DNA within nuclei in the midguts of imidacloprid-treated P24 and P48 individuals. By contrast, this was not observed in labelled cells in the control, which precluded any statistical comparisons among treatments (Fig. 6).

Discussion

The present study analysed the toxic effects of imidacloprid on survival and in the midguts of *St. aegypti.* The sub-lethal effects of imidacloprid in the midguts of *St. aegypti* occurred initially in the anterior region of the midguts in L4 individuals, with deformed nuclei in digestive cells and the presence of large spaces representing dead cells. Throughout development, the effects of imidacloprid progressed to the posterior region in P24 individuals. These changes may have resulted from interference in the regenerative cell proliferation that occurs with the onset of cell death in the anterior midgut of L4 individuals shortly before moulting (Nishiura *et al.*, 2003; Ray *et al.*, 2007; Fernandes *et al.*, 2014), which would suggest that digestive cells that died after imidacloprid treatment were not replaced during metamorphosis. Thus, the data suggest that the lethal effect promoted by the neurotoxic mechanism of imidacloprid affected the development of the *St. aegypti* midgut.

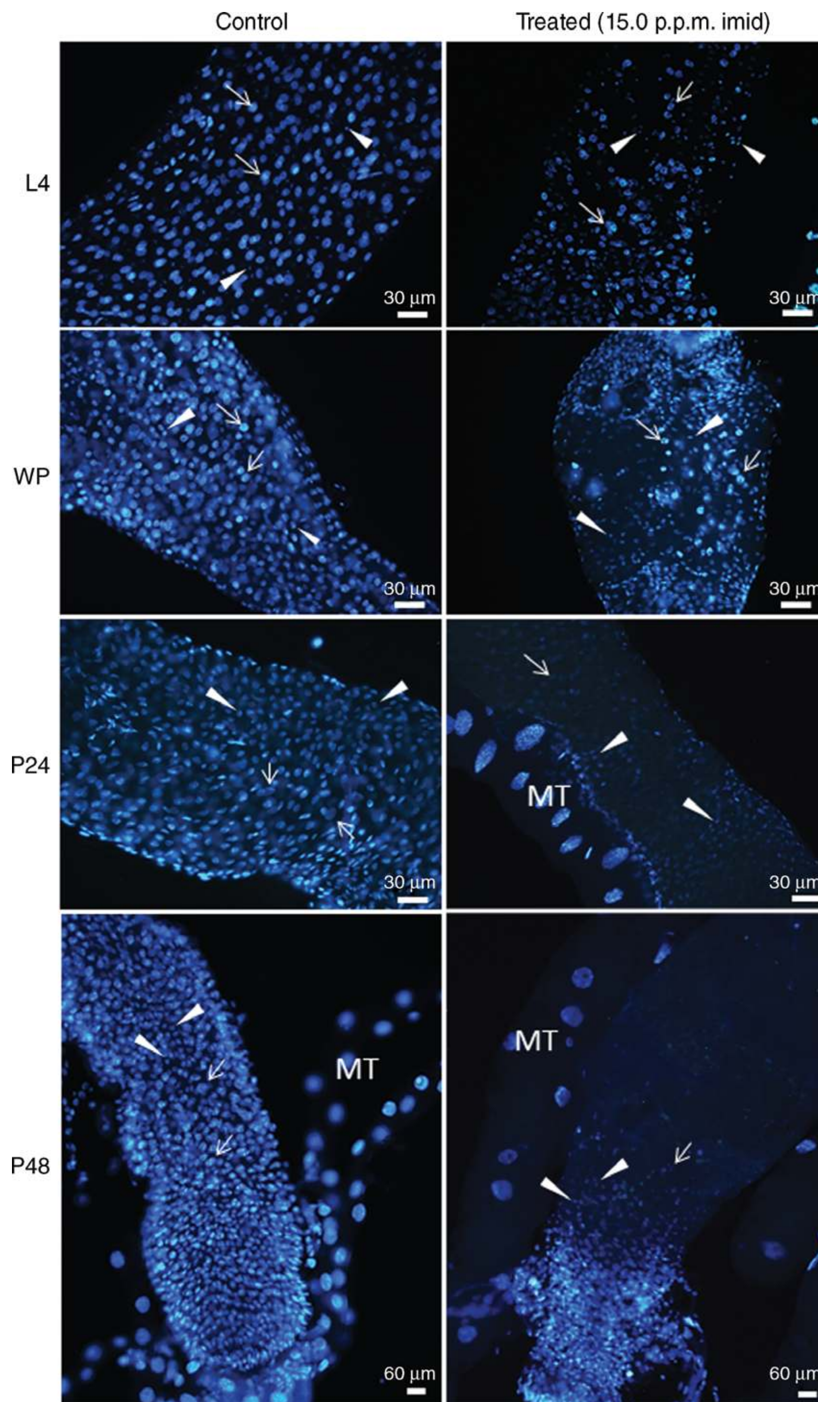

Fig. 1. Whole mounts of the midgut of *Stegomyia aegypti* [fourth instar larvae (L4), white pupae (WP), and pupae collected 24 h (P24) and 48 h (P48) after ecdysis] without (Control) or with (Treated) 15.0 p.p.m. imidacloprid. Cell nuclei were stained with DAPI (blue). Arrows indicate digestive/differentiating digestive cell nuclei; arrowheads indicate regenerative cell nuclei; MT, Malpighian tubules.

The number of differentiating digestive cells increased, whereas the number of regenerative cells decreased, at the P24 stage in control individuals. This can be explained by the occurrence of cell differentiation, whereby the regenerative cells are 'consumed' by becoming new digestive cells in adults (Nishiura & Smouse, 2002; Nishiura *et al.*, 2003). In individuals treated with imidacloprid, the digestive cells (or differentiating cells) occurred in lower numbers from L4 to NE, whereas the number of regenerative cells remained constant, indicating that the regenerative cells of treated individuals did not differentiate, and thereby interfering with the remodelling of the midgut epithelium. Additionally, the reduced number of regenerative cells in treated L4 individuals may reflect the decreased proliferation of these cells that occurs after the passage from L3 to L4 in *St. aegypti* (Ray *et al.*, 2007).

The degeneration of digestive cells begins at the end of the L4 stage and ends 18 h after pupation (Nishiura *et al.*, 2003; Ray *et al.*, 2007). Because the digestive cells of the L4 and WP stages

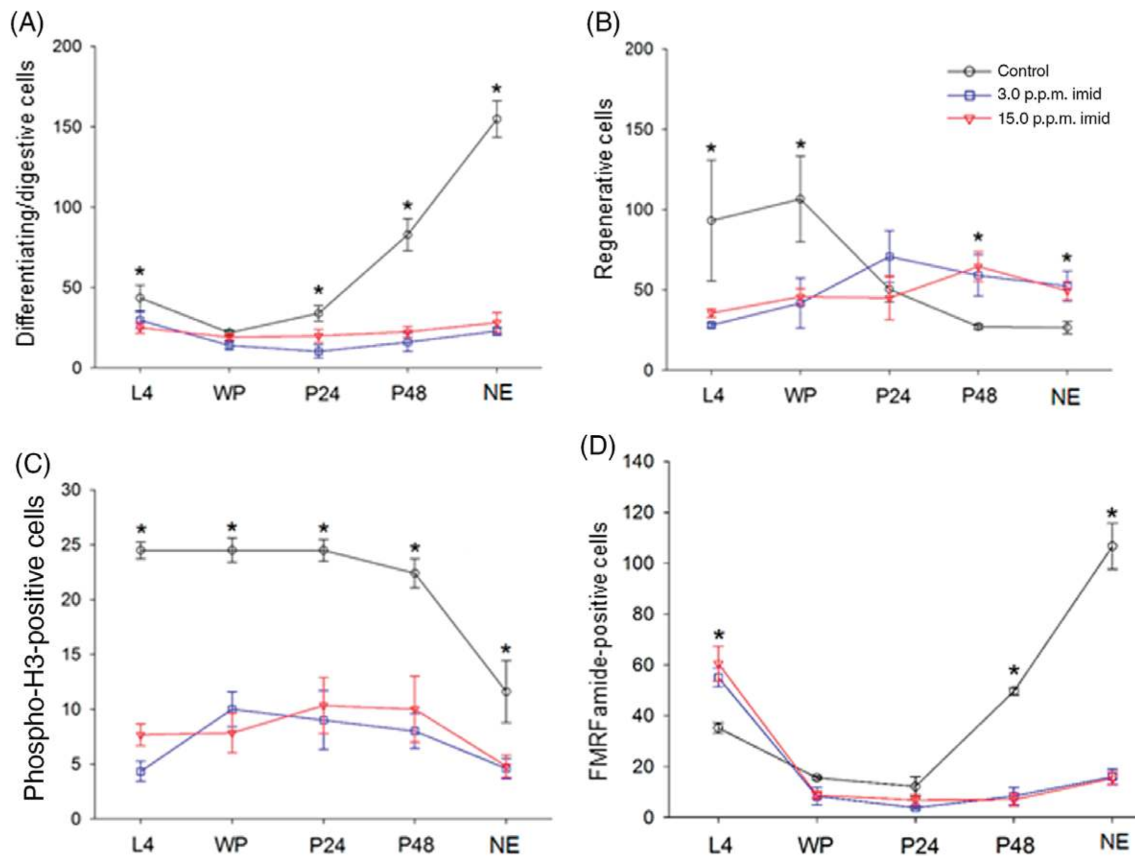

Fig. 2. Numbers of (A) digestive cells, (B) regenerative cells, (C) mitotic (phospho-H3-positive) cells and (D) enteroendocrine/FMRF-positive cells in the midgut of *Stegomyia aegypti* at different developmental stages/phases [fourth instar larvae (L4), white pupae (WP), pupae collected 24 h (P24) and 48 h (P48) after ecdysis, and newly emerged adult females (NE)]. Individuals were treated with 3.0 or 15.0 p.p.m. imidacloprid. Vertical bars represent standard deviations; * indicates a significant difference ($P < 0.05$).

are typically in the process of cell death (Ray *et al.*, 2007; Fernandes *et al.*, 2014), it was not possible to identify the effects of imidacloprid in larval digestive cells during these stages. In P24 control individuals, many differentiating cells were present, giving rise to new digestive cells (Nishiura *et al.*, 2003; Ray *et al.*, 2007; Fernandes *et al.*, 2014). The number of differentiating cells was lower in individuals exposed to imidacloprid, and these cells had highly vacuolated cytoplasm and disrupted membranes. The features of cell death were also found in NE and P48 individuals, indicating that the cytotoxic effect of imidacloprid persisted throughout development, impairing the reconstruction of the digestive epithelium during the metamorphosis of *St. aegypti*.

Typically, the degeneration of larval digestive cells begins in the anterior midgut at the L4–pupal transition and continues until 18 h after the onset of pupation (Nishiura & Smouse, 2002; Nishiura *et al.*, 2003). However, in individuals exposed to imidacloprid, TUNEL-positive cells, indicating regenerative and digestive/differentiating cells, were present after the end of the degeneration of larval digestive cells. This suggests that the cytotoxic effects of imidacloprid began in the regenerative cells at the L4 stage and negatively interfered with the restructuring of the midgut of *St. aegypti*.

The proliferation of regenerative cells occurred in a coordinated manner during the remodelling of the midgut of *St. aegypti*, with regions of proliferation starting at the anterior midgut region during L4 and progressing to the posterior midgut region during NE (Fernandes *et al.*, 2014). Imidacloprid reduced the number of H3-positive cells (i.e. the number of proliferating cells) during remodelling of the midgut; this may compromise metamorphosis and interfere with the maintenance of the midgut throughout the life of the adult mosquito. Although imidacloprid acts agonistically on nicotinic acetylcholine receptors (nAChRs) in neurons (Casida & Quistad, 2004), its interference with the proliferation of regenerative cells occurs through an unknown path.

Interestingly, endocrine (FMRF-positive) cells were present in all stages of *St. aegypti* development; however, these cells were localized to the posterior region of the midgut (Moffett & Moffett, 2005). Similar to digestive cells, endocrine cells originate through the differentiation of regenerative cells (Ohlstein & Spradling, 2006), which presumably appear *de novo* from the P24 stage (Fernandes *et al.*, 2014). However, the number of endocrine cells in L4 individuals treated with imidacloprid was higher than that of the control, and the endocrine cells were localized in the anterior midgut. These characteristics

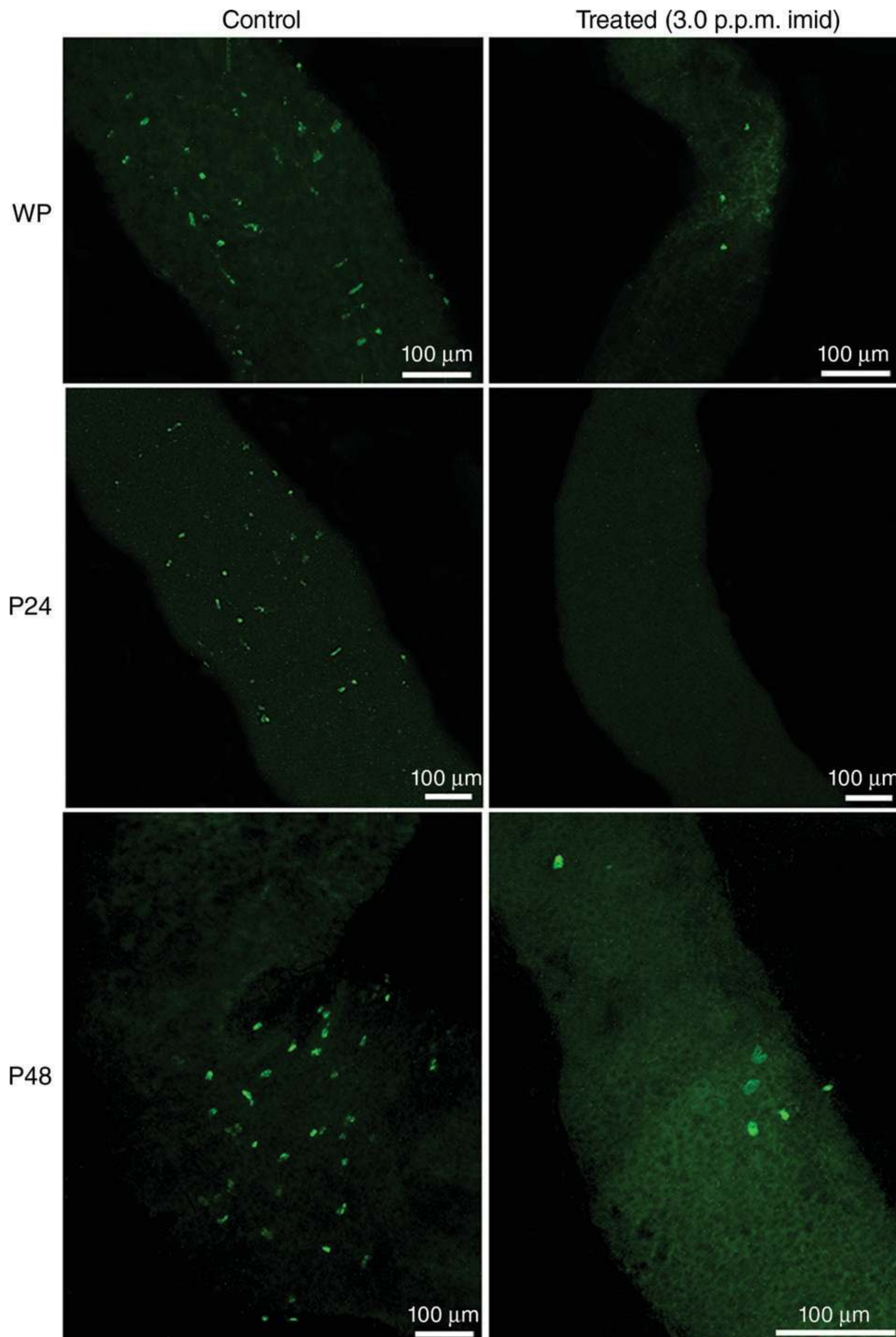

Fig. 3. Dividing cells (phospho-H3-positive) in the midguts of *Stegomyia aegypti* at different developmental stages [fourth instar larvae (L4), and pupae collected 24 h (P24) and 48 h (P48) after ecdysis] in control and treated (3.0 p.p.m. imidacloprid) groups.

suggest that endocrine cells served to ameliorate the toxic effects of the insecticide through secretion of modulatory peptides, stimulating cell proliferation (Moffett & Moffett, 2005). Moreover, the number of endocrine cells did not increase after metamorphosis in the midgut of treated individuals, possibly as a result of the impairment of regenerative cell differentiation. Importantly, reduced numbers of endocrine cells can be harmful to the midgut of adult *St. aegypti* because these cells are critical for digestion (Brown *et al.*, 1986; Moffett & Moffett, 2005).

Imidacloprid and other insecticides belonging to the neonicotinoid class act as potent, partial or full agonists at nAChRs in the insect central nervous system (Tomizawa & Casida, 2005). The binding of imidacloprid on nAChRs causes persistent

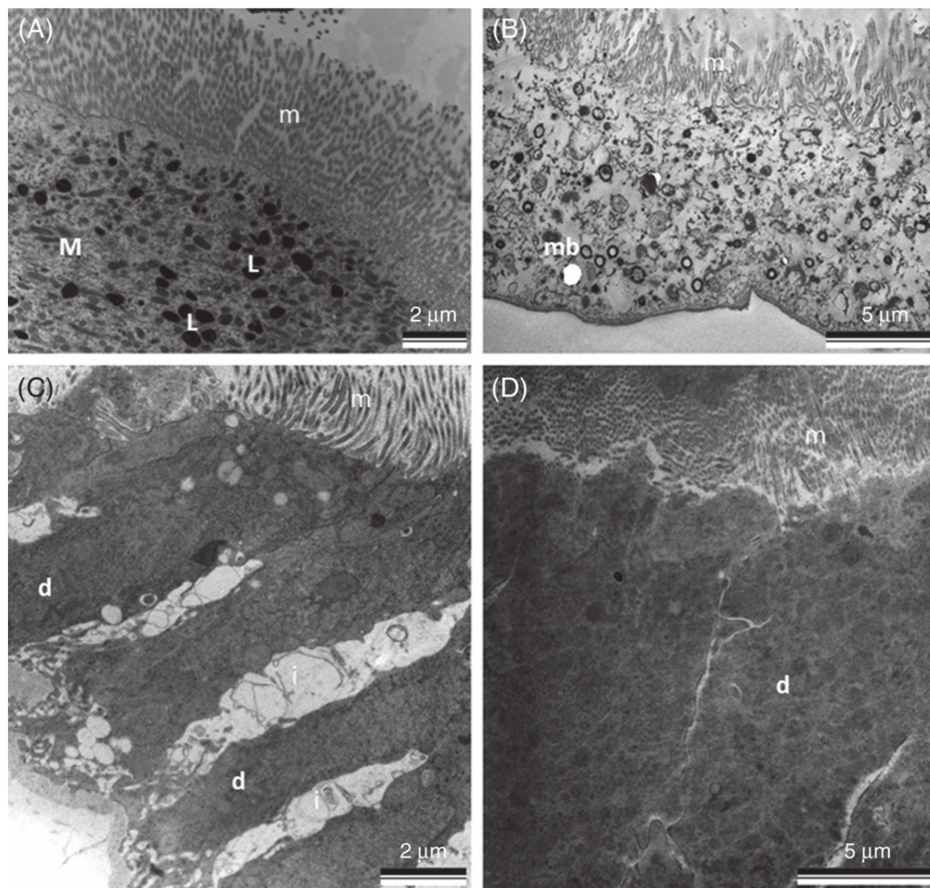

Fig. 4. Electron micrographs of the midguts of untreated (control) *Stegomyia aegypti*. (A) Apical region of digestive cell with developed microvilli (m) in fourth instar larvae (L4). Several mitochondria (M) and lysosome-like structures (L) are shown in the cell apex. (B) Digestive cells in the degeneration process with an electron-lucent cytosol, degenerating microvilli (m), and several multilamellar bodies (mb) in white pupae (WP). (C) Differentiating electron-dense digestive cells (d) in pupae at 24 h after ecdysis (P24), showing intercellular spaces (i) and microvilli (m). (D) Columnar digestive cells (d) in pupae at 48 h after ecdysis (P48).

activation, which leads to increased excitatory responses and insect death (Casida & Quistad, 2004). In insects, nAChRs are widely distributed only in the central nervous system (brain), whereas glutamate receptors are predominant at neuromuscular junctions (Breer & Sattelle, 1987). It is proposed that mortality found mainly at the larval stage was induced by the activation and collapse of cholinergic synapses within the central nervous system. However, disorders of the midgut can be a consequence of direct stress within the cells, regardless of their effects on the nervous system. Although sub-lethal effects on the midgut are not the primary cause of mortality during immature stages, these effects can be dangerous to adult mosquitoes because they affect the absorption of nutrients, and thus longevity and reproduction, particularly because blood feeding is crucial to egg development.

The present data show that imidacloprid led to mortality and additional sub-lethal effects in the post-embryonic development of the midgut of *St. aegypti*. Imidacloprid affected the remodelling of the midgut of *St. aegypti* by inhibiting the proliferation and differentiation of regenerative cells. Finally, the results suggest that the insecticide has the potential to control mosquito populations because, even in individuals that survived to adulthood, midgut epithelium malformation was observed.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/mve.12122

Figure S1. Enteroendocrine/FMRFamide-positive cells (green) in the anterior–mid region of the midguts of untreated fourth instar larvae of *Stegomyia aegypti*.

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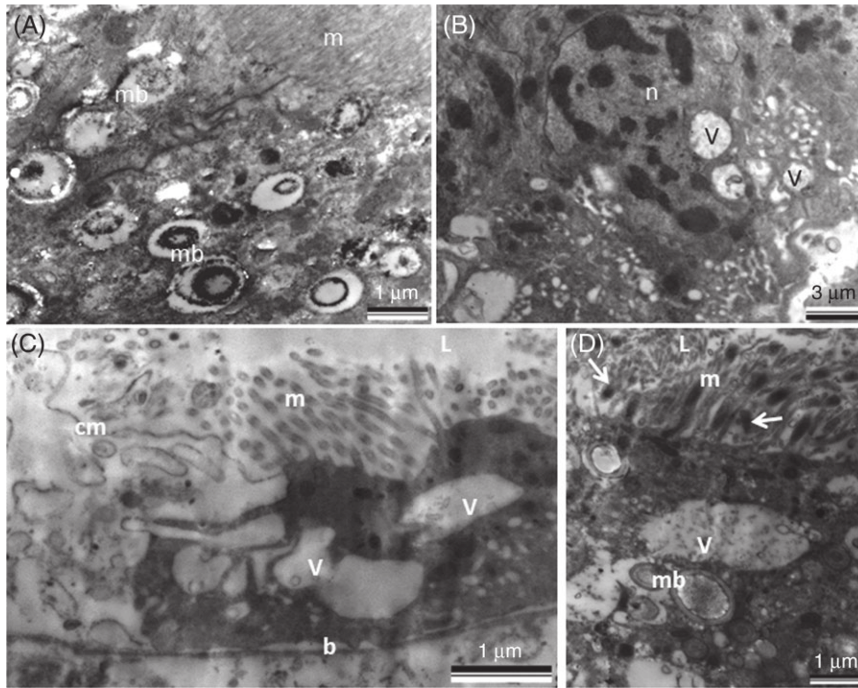

Fig. 5. Electron micrographs of the midgut of *Stegomyia aegypti* treated with 3.0 p.p.m. imidacloprid. (A) Apical region of the digestive cells in fourth instar larvae (L4) with multilamellar bodies (mb) in the cell apex. (B) Differentiating digestive cells with a deformed nucleus (n) and large electron-lucent vacuoles (V) occupying the cytoplasm in white pupae (WP). (C) Differentiating cells with disrupted cell membrane (cm) and electron-lucent vacuoles (V) in pupae at 24 h after ecdysis (P24), showing basal lamina (b), midgut lumen (L) and microvilli (m). (D) Digestive/differentiating cells with multilamellar bodies (mb) and electron-lucent vesicles (V) pupae at 48 h after ecdysis (P48). Mitochondria (arrowheads) are seen in the apical region within microvilli and in the lumen (L).

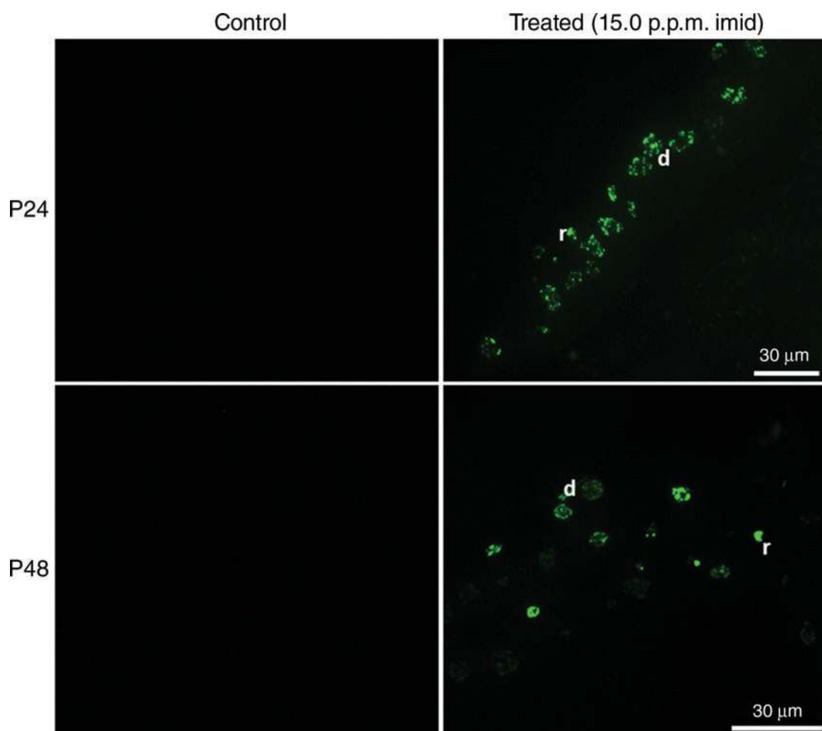

Fig. 6. Cells positive for TUNEL reaction in the midguts of *Stegomyia aegypti* exposed to 15.0 p.p.m. imidacloprid. Digestive (d) and regenerative (r) cell nuclei (green) with fragmented DNA are shown in pupae at 24 h (P24) and 48 h (P48) after ecdysis without (Control) or with (Treated) 15.0 p.p.m. imidacloprid.

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