Treatment of an *Aedes aegypti* colony with the Cry11Aa toxin for 54 generations results in the development of resistance

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To study the potential for the emergence of resistance in Aedes aegypti *populations, a wild colony was subjected to selective pressure with Cry11Aa, one of four endotoxins that compose the* Bacillus thuringiensis *serovar* israelensis *toxin. This bacterium is the base component of the most important biopesticide used in the control of mosquitoes worldwide. After 54 generations of selection, significant resistance levels were observed. At the beginning of the selection experiment, the half lethal concentration was 26.3 ng/mL and had risen to 345.6 ng/mL by generation 54. The highest rate of resistance, 13.1, was detected in the 54th generation. Because digestive proteases play a key role in the processing and activation of* B. thuringiensis *toxin, we analysed the involvement of insect gut proteases in resistance to the Cry11Aa* B. thuringiensis *serovar* israelensis *toxin. The protease activity from larval gut extracts from the Cry11Aa resistant population was lower than that of the* B. thuringiensis *serovar* israelensis *susceptible colony. We suggest that differences in protoxin proteolysis could contribute to the resistance of this* Ae. aegypti *colony.*

Key words: *Bacillus thuringiensis* - *Aedes aegypti* - Cry11Aa toxin resistance - midgut proteases

Due to the increased resistance of mosquitoes to chemical pesticides and the risks these chemicals pose to humans and the environment, insect control using bacteria, such as *Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis*, has increased in various regions of the world over the last three decades, with a market growth prediction of up to 20% of the world's pesticide use by 2020 (Whalon & Wingerd 2003, Kumar et al. 2008). The use of insecticides based on *B. sphaericus* toxins have achieved moderate commercial success in developed countries, but their high cost discourages their use in many developing countries. Moreover, their long-term use in *Culex quinquefasciatus* and *Culex pipiens* control programs underway in France, India, Brazil and China has resulted in resistance in the mosquito populations (Sinègre et al. 1994, Rao et al. 1995, Silva-Filha et al. 1995, Yuan et al. 2000, Nielsen-Leroux et al. 2002). Although the use of insecticides based on *B. thuringiensis* serovar *israelensis*, which have a broader spectrum of activity against species of *Aedes*, *Culex* and *Anopheles,* has proven to be very effective, their activity in the field is limited because it is affected by various biological and environmental factors and therefore requires frequent application (Mittal 2003). The activity of *B. thuringiensis* serovar *israelensis* is based in the synergistic effects of Cry and Cyt toxins (Crickmore et al. 1995) and reaches half lethal concentration (LC_{50}) values between 2-30 ng/mL, depending on the mosquito species and age of the insects (Orduz et al. 1998, Boujelida et al. 2008). Cry4A, Cry4B and Cry11A toxins, which range in size from 68-135 kDa, contribute to the toxicity of intact crystals in a synergistic manner. The related toxin CytA is haemolytic and cytolytic in vitro and is specifically active against dipteran larvae in vivo (Crickmore 1995).

Despite the potency of the products based on *B. thuringiensis* serovar *israelensis*, the efficacy of these types of biopesticides has a limitation: the crystals settle to the base of the water column, away from the larval feeding range, within a few days of application. Alternatives have been proposed to overcome this problem, such as the development of live recombinant algae or bacteria that express toxin(s) and remain within the feeding range of mosquito larvae (Romero et al. 2001, Khasdan et al. 2003, Zheng et al. 2007). However, an important concern raised against this approach is the high risk of the development of resistance by the target insects as resistance to individual *B. thuringiensis* serovar *israelensis* toxins has been observed in laboratory colonies of *Cx. quinquefasciatus.* In one selection experiment in which individual toxins or combinations of toxins from *B. thuringiensis* serovar *israelensis* were used on laboratory colonies of *Cx. quinquefasciatus*, Georghiou and Wirth (1997) observed that lower levels of resistance occurred when all the toxins were used in combination.

The mechanisms of insect resistance to *B. thuringiensis* fall into several categories: altered binding of Cry toxins to receptors in the midgut, alterations in the proteolytic processing of the Cry toxin (decreased rates of activation or increased rates of toxin degradation), elevated immune response or enhanced esterase production (Oppert et al. 1997, Bravo et al. 2007, Bravo & Soberon 2008) and rapid regeneration of the damaged midgut epithelium, which prevents septicaemia (Ferré & Van Rie 2002). Recently, Bonin et al. (2009) have shown that the resistance of a natural *Ae. aegypti* population to *B. thuringiensis* serovar *israelensis* toxins was associated with a polymorphism in

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the cadherin gene. In addition to this polymorphism, the under-expression of this gene was observed. Here, our objective was to determine under laboratory conditions the risk of the development of toxin resistance in *Ae. aegypti* colonies subjected to the continuous selective pressure of the Cry11A toxin from *B. thuringiensis* serovar *israelensis*. In this paper, we suggest that differences in the proteolytic activity of mosquito larvae midgut enzymes could be responsible for the resistance we observed in this *Ae. aegypti* colony.

MATERIALS AND METHODS

Insect strains - To ensure the genetic diversity of the *Ae. aegypti* colony to be treated with the Cry11Aa toxin, an artificial colony was established by mixing individuals collected from the urban zones of Medellín (state of Antioquia), Riohacha (state of Guajira), San Andres (state of San Andres) and Valledupar (state of Cesar), Colombia and individuals from the reference standard Rockefeller strain, kindly provided by Dr Gary Clark (Notre Dame University, Indiana, USA). *B. thuringiensis*-based products had not been previously applied at any of the locations where mosquitoes were collected. Five hundred adults from each location were used to establish the initial colony, which was maintained in quarantine for five generations before the start of the selection experiments. In each of the quarantine generations, between 10,000-15,000 larvae were reared. Prior to the start of the selection experiments, the colony was divided into two groups: one group was kept under continuous selection with Cry11Aa (AaOGr) and the other group was not exposed to selection and was used as a control (AaOGs). Both colonies were maintained in the insectary at 30ºC with 70-80% relative humidity and a light:dark photoperiod of 12:12 h. The colonies were reared on an artificial diet under laboratory conditions and located in different rooms that were separated by four doors.

Cry11Aa toxin solution preparation - The Cry11Aa toxin used in this study was obtained from the *B. thuringiensis* strain 4Q2-81, carrying the plasmid pHT640 encoding the *cry11Aa* gene (Poncet et al. 1993). Toxin production was performed in a 20-l volume fermenter under the following conditions: air, 18 lpm, temperature, 30ºC, and agitation speed, 300 rpm for 72 h using a culture medium described by Liu and Bajpai (1995) with modifications (0.5 g/L KH_2PO_4 and 0.5 g/L K_2HPO_4). The final whole culture (FWC) was concentrated using a tangential flow filtration system equipped with 0.22 µm filter cassettes (Millipore, US).

The spore-crystal suspension was incubated for 30 min with 1 M NaCl and then washed twice with distilled water. The concentrated FWC was centrifuged, resuspended in phosphate buffered saline (PBS) (0.24 g KH_2PO_4, 1.44 g Na_2HPO_4, 8 g NaCl, 0.20 g KCl, 800 mL H_2O) and dried in a mini spray dryer (Büchi) to obtain a final yield of 19.5 g of spore-crystal technical powder, which was stored at -20ºC until use.

Bioassay procedures and selection experiments - To determine the baseline susceptibility of the initial AaOG colony to the Cry11Aa toxin and for larval resistance selection experiments, the technical powder was suspended in PBS and homogenised with glass beads. These suspensions were analysed using the Bradford method to determine protein concentration and crystal-spore suspensions were maintained at 4ºC and used within 10 days (Armengol et al. 2006). Cry11Aa toxin stock suspensions with protein concentrations of 20, 200 and 2,000 µg/mL were generated and bioassays to determine the LC_{50} were performed in 237 mL plastic cups containing 100 mL of tap water and 20 early third instar *Ae. aegypti* larvae. A minimum of five concentrations were tested in triplicate, with mortality rates ranging from 2-98%. The experiment was performed on three different days and mortality was evaluated after 24 h of treatment. The results were submitted for probit analysis (Raymond 1995) to determine the LC_{50}. This procedure was performed every third generation on both the Cry11Aa-treated colony (AaOGr) and the untreated colony (AaOGs). To select resistant colonies, 1,000 early third instar larvae were cultured in pans containing 2 L water combined with the appropriate concentration of Cry11Aa toxin. Mosquito larvae that survived after 24 h were recovered, rinsed with water, counted and transferred to a clean pan with 2 L water supplemented with an artificial diet. To obtain at least 1,000 surviving adults to initiate the subsequent generation, selective pressure was placed on 5,000-20,000 larvae per generation. The toxin dosage was adjusted to obtain 75-85% mortality each generation. Exposure to the Cry11Aa toxin was applied for 54 consecutive generations. The parallel untreated *Ae. aegypti* colony, AaOGs, likewise derived from the initial colony, was maintained under the same conditions without Cry11Aa toxin treatment. The adult mosquitoes were placed in cages and fed 10% sugar water. Blood from adult mice served as food for adult females.

Resistance to the Cry11Aa toxin was expressed as a resistant ratio (RR), which is the ratio of the LC_{50} of the selected strain (AaOGr) to that of the unselected strain (AaOGs). For RR1, the LC_{50} of the AaOGr colony in a given generation was divided by the LC_{50} of the initial AaOGs generation (Georghiou & Wirth 1997). For RR2, the LC_{50} of the AaOGr colony in a given generation was divided by the LC_{50} of individuals from the AaOGs colony of the same generation.

Aedes aegypti third instar larvae mortality kinetics - To study the mortality kinetics of both *Ae. aegypti* colonies, 20 early third instar larvae from generation 54 were placed in 100 mL of dechlorinated water with 500 times the initial LC_{50} of Cry11Aa (26.3 ng/mL). Larvae incubated in water with no added Cry11Aa acted as the negative control. The bioassays were incubated at 30ºC and mortality was scored every 10 min for 300 min. The bioassays were performed in triplicate and repeated on three different days (Orduz et al. 1994).

Mosquito larvae midgut protease activity - One hundred *Ae. aegypti* third instar larvae from generation 54 from both the AaOGr and AaOGs colonies were washed and placed on ice for 15 min. For each specimen, the gut was excised from the larval body, excluding the peritrophic membrane, immediately submersed in ice-cold TM buffer (100 mM Tris-HCl, pH 7.2, 300 mM mannitol) and homogenised with a syringe fitted with

a 21-gauge needle. The homogenates were centrifuged at 12,000 rpm for 30 min at 4°C and the supernatants were carefully separated from the pellet. Protein in the supernatant, referred to subsequently as the gut extract, was quantified using the Bradford method and aliquots were stored at -70ºC until use (Segura et al. 2000).

To determine the total protease activity, gut extracts from insects of both colonies (0.5 µg of total protein) were subjected to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis co-polymerised with 0.1% gelatin (zymogram analysis) at 4ºC under reducing conditions without previously boiling the samples, as described by Hames and Rickwood (1990). After electrophoresis, the gels were washed four times with 250 mM Tris-HCl, 2% Triton X-100 buffer (pH 7) at 4ºC and incubated in 50 mM Tris-HCl buffer (pH 7) and 50 mM carbonate buffer ($NaHCO_3$-Na_2HPO_4, pH 10.3) for 1 h at 37ºC. The bands were visualised using Coomassie blue staining. The inhibition of protease activity in the gel was assayed using a mixture of phenylmethylsulfonyl fluoride (PMSF), N-p-tosyl-L-lysine chloromethyl ketone (TLCK) and N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) to a final concentration of 1 mM of each one. All reagents were obtained from Sigma (St. Louis, MO, USA).

To determine the proteolytic activities of gut extract trypsin and chymotrypsin, samples of gut extracts from insects of both colonies were thawed and spun at 12,000 rpm for 2 min and the supernatants containing soluble gut enzymes were tested using 96-well microplate assays. To determine the trypsin activity, samples containing 50 µg/mL protein were diluted 1:100 in two buffers [buffer A (50 mM Tris-HCl, pH 7.0, 20 mM $CaCl_2$) and buffer B (50 mM $NaHCO_3$-Na_2CO_3, pH 10.3] and 50 µL was added to the well of a microplate. To initiate the reaction, *N*-α-benzoyl-L-arginine *p*-nitroanilide (Sigma) (100 mg/mL in dimethyl sulfoxide) was diluted 1:100 in buffer A or B and 50 µL was added to each sample well for a final concentration of 1.15 mM. After 30 s incubation at 37ºC, the absorbance was monitored at 405 nm at 15 s intervals over a 5 min period. The change in absorbance per min was calculated using a kinetic module (Biorad 680-XR Microplate reader) and the data were compared for each gut extract. N-succinyl-ala-ala-pro-phe-p-nitroanilide (Sigma) (100 mg/mL in dimethyl sulfoxide) was used as a substrate to determine the chymotrypsin activity of the *Ae. aegypti* midgut and the assay was performed as described above. Each test was performed in triplicate (Oppert et al. 1997).

RESULTS

Development of Ae. aegypti resistance - The Cry11Aa LC_{50} of the initial *Ae. aegypti* colony was 26.3 ng/mL (22-30 ng/mL, 95% confidence interval). This dose resulted in 15-25% survival 24 h after the removal of early fourth instar larvae from the toxin solution and these survivors initiated the first generation of the AaOGr colony.

Tolerance to Cry11Aa began to increase slightly in the AaOGr colony from the 3rd-24th generations (RR1 = 0.8-2.3, RR2 = 1.0-2.4) and a further small increase in tolerance was observed from the 27th-36th generation (RR1 = 2.3-3.2, RR2 = 2.8-3.1). Finally, from the 36th-54th generations, a steady increase above 5 was observed in the RR (RR1 = 5.6-13.1, RR2 = 5.5-10.1). These results indicate that, under the conditions used in these experiments, it is possible for *Ae. aegypti* mosquitoes to develop resistance to the *B. thuringiensis* Cry11Aa toxin (Table).

Mortality kinetics - Third instar larvae from generation 54 of both AaOG colonies were challenged with 500 times the Cry11Aa LC_{50} of the initial *Ae. aegypti* colony (13.15 mg/mL) to examine additional differences between the *Ae. aegypti* colonies. Mortality for the AaOGs larvae first occurred after 110 min (5%), reaching 50% after 220 min and 90% after 300 min, at which point the assay was terminated. On the contrary, mortality for the AaOGr larvae did not exceed 10% after 300 min (Fig. 1).

Zymogram analysis of mosquito larvae gut extracts - A zymogram analysis was used to assess the relative number of soluble gut proteinases in the extracts from Cry11Aa-susceptible and Cry11Aa -resistant *Ae. aegypti* colonies. Three bands were observed between 45-70 kDa with gelatinolytic activity in the gut extracts from both mosquito colonies (Fig. 2). However, the intensity of the AaOGr bands was fainter, suggesting that the protease activity of the gut extracts from this group was lower (Fig. 2, Lane 3). Additionally, the effect of serine protease inhibitors was tested. PMSF, TPCK and TLCK inhibitors used in combination significantly inhibited the protease activity of the gut extracts, suggesting that serine proteases mediate the primary gelatinolytic activity (Fig. 2, Lanes 4, 5).

TABLE

Variation in the susceptibility of *Aedes aegypti* larvae subjected to continuous selection pressure with *Bacillus thuringiensis* serovar *israelensis* Cry11Aa toxin for 54 consecutive generations

Generations under selection	LC_{50}	95% CI	RR1[a]	RR2[b]
AaOG	26.3	22.5 < LC < 30.1	1.0	1.0
AaOGr3	31.3	27.1 < LC < 35.4	1.2	1.7
AaOGr6	24.9	21.5 < LC < 28.2	0.9	1.8
AaOGr9	39.2	32.8 < LC < 46.6	1.5	1.7
AaOGr12	34.7	30.9 < LC < 38.5	1.3	2.4
AaOGr15	60.1	50.6 < LC < 71.3	2.3	1.7
AaOGr18	37.7	31.3 < LC < 43.0	1.4	1.6
AaOGr21	28.1	22.0 < LC < 32.5	1.1	1.8
AaOGr24	21.0	3.0 < LC < 33.5	0.8	1.6
AaOGr27	59.6	42.8 < LC < 110.3	2.3	2.8
AaOGr30	68.2	58.7 < LC < 79.9	2.6	2.8
AaOGr33	84.6	67.3 < LC < 127.5	3.2	3.1
AaOGr36	72.0	62.6 < LC < 86.0	2.7	2.8
AaOGr39	162.0	133.2 < LC < 235.8	6.6	5.9
AaOGr42	293.88	234.9 < LC < 410.3	11.1	7.5
AaOGr45	253.0	214.4 < LC < 345.8	9.6	9.0
AaOGr48	296.0	251.2 < LC < 379.4	11.3	9.8
AaOGr51	146.4	110.1 < LC < 237.8	5.6	5.5
AaOGr54	345.6	260.8 < LC < 460.23	13.1	10.1

a: compared to the first generation before exposure to the Cry11Aa toxin: *b*: compared to the contemporary untreated generation; CI: confidence interval; $LC_{50:}$ half lethal concentration; RR: rate of resistance.

Determination of proteases activities – The trypsin and chymotrypsin activities of the gut extracts of *B. thuringiensis*-susceptible and -resistant *Ae. aegypti* colonies were compared in buffers of different pH (7.0 and 10.3). The trypsin activity from susceptible and resistant colonies decreased approximately six and four fold, respectively, at pH 7.0 compared to pH 10.3. Likewise, the chymotrypsin activity of susceptible and resistant colonies decreased nine and six fold, respectively, at pH 7.0 compared to pH 10.3 (Fig. 3). In addition, the relative activities of both midgut serine proteases, trypsin and chymotrypsin, in the *Ae. aegypti* resistant colony, were 28% and 37%, respectively, of those of the susceptible colony at pH 10.3 (Fig. 3C, D).

DISCUSSION

In this study, we report that after 54 generations of exposure to the Cry11Aa toxin of *B. thuringiensis* serovar *israelensis*, an *Ae. aegypti* colony developed significant toxin resistance. The treated colony had a very low mortality rate compared to that of the unselected colony when exposed to 500 times the initial Cry11A LC_{50} toxin, and midgut protease analysis indicated that the activities of trypsin and chymotrypsin in the midguts of insects in the Cry11A-treated colony had decreased.

The significant resistance of the AaOGr colony to the Cry11Aa toxin was not observed until generation 39, although a steady increase in tolerance to the Cry11Aa toxin was observed starting with generation 27. Georghiou and Wirth (1997) generated several colonies of the mosquito *Cx. quinquefasciatus* that were resistant to one of several toxins of *B. thuringiensis* serovar *israelensis*. These colonies reached RRs between 2-42.9 within 28 generations at LC_{50} and RRs decreased when a combination of toxins was used (Cry4A+4B+11A+Cyt1A) and increased when the toxin Cry11A was used alone. Wirth et al. (2004) used *B. thuringiensis* subsp. *jegathesan* to generate a resistant colony of *Cx. quinquefasciatus*. The selected colony developed 13-fold resistance in 22 generations, but this resistance dropped to 2.3 fold by generation 26 and remained low. However, when isolated Cry11B toxin from this bacterium was used, resistance was detected by generation 18 and reached a maximum of 38 fold that was sustained through generation 40.

Recently, the persistence of *B. thuringiensis* serovar *israelensis* spread by humans in a wild mosquito habitat was reported in Switzerland (Tilquin et al. 2008). Furthermore, a resistant colony of *Ae. aegypti* was generated experimentally following 18 generations of continuous exposure to toxic leaf litter material collected from natural mosquito breeding ponds. When the *Ae. Aegypti*-resistant colony was challenged with individual Cr4A, Cry4B, Cry11A and Cyt 1A toxins, the RRs were 30.2, 13.7, 6.3 and 3.0, respectively (Bonin et al. 2009). The exposure of *Ae. aegypti* larvae to toxins at 500-fold the LC_{50} of three mosquitocidal strains of *B. thuringiensis* (*israelensis*, *jegathesan* and *medelllin*) caused at least 90% mortality within 60 min (Orduz et al. 1994). Here, *Ae. aegypti* larvae from generation 54 that were exposed to 500 times the Cry11A LC_{50} (13.15 mg/mL) showed different patterns in mortality kinet-

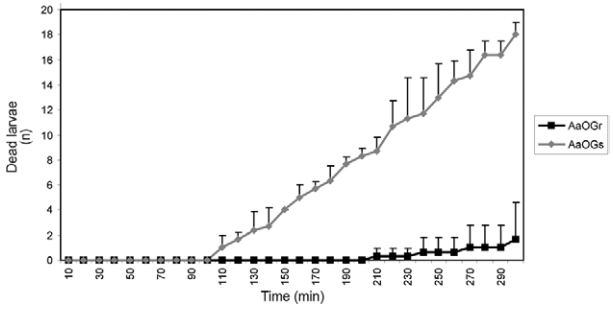

Fig. 1: mortality kinetics of *Aedes aegypti* third instar larvae from generation 54 selected for resistance to Cry11A toxin compared with the mortality of the contemporary susceptible *Ae. aegypti* untreated colony. AaOGr: selected strain; AaOGs: unselected strain.

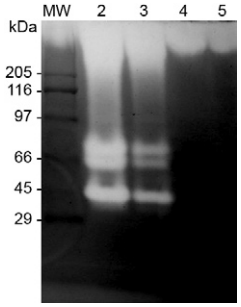

Fig. 2: zymogram analysis of gut extracts of *Aedes aegypti* colonies. Total protein (0.5 µg) was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis co-polymerized with 0.15% gelatin. Lanes 2, 3 were incubated without mix inhibitors and 4, 5 were incubated in presence to phenylmethylsulfonyl fluoride, N-p-tosyl-L-lysine chloromethyl ketone and N-p-tosyl-L-phenylalanine chloromethyl ketone mix inhibitors. Lanes 2, 4: AaOGs colony (unselected strain); 3, 5: AaOGr colony (selected strain); MW: molecular weight.

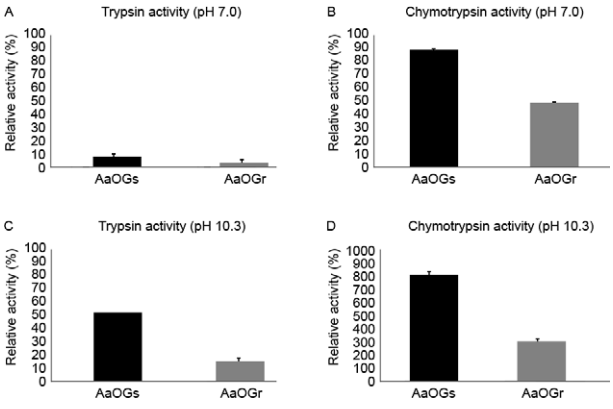

Fig. 3: relative activity of trypsin (A-C) and chymotrypsin (B-D) from *Aedes aegypti* AaOGs (unselected strain) and AaoGr (selected strain) larvae using the substrates *N*-α-benzoyl-L-arginine *p*-nitroanilide and N-succinyl-ala-ala-pro-phe-p-nitroanilide at pH 7.0 and 10.3, respectively. Bars represent the standard deviation from two independent experiments by triplicate.

ics. In the AaOGs colony, 90% of the treated larvae were dead 300 min after exposure to the Cryl1A toxin; meanwhile, only 10% of the mosquito larvae from the AaOGr colony were dead in the same time, demonstrating a completely different susceptibility to the Cryl1A toxin. It is possible that the differences in mortality rates following treatment with wild-type strains of *B. thuringiensis* serovar *israelensis* (Orduz et al. 1994) compared to treatment with purified Cryl1Aa toxin are due to the synergy that has been reported between the *B. thuringiensis* serovar *israelensis* toxins (Tabashnik 1994).

Several instances of resistance to *B. thuringiensis* toxins have been described in Lepidoptera (*Plutella xylostella*, *Plodia interpunctella* and *Trichoplusia ni*), Coleoptera (*Leptinotarsa decemlineata* and *Chrysomela scripta*) and Diptera (*Cx. quinquefasciatus*) species under laboratory, glass house or field conditions, and the strategies of resistance have been determined to include both biochemical and genetic mechanisms (reviewed by Ferré & Van Rie 2002).

In the case of the *Cx. quinquefasciatus* colonies resistant to *B. thuringiensis* serovar *israelensis*, Wirth et al. (2010) concluded that the pattern of the inheritance of resistance to the Cryl1Aa toxin was autosomal and that F1 individuals displayed intermediate resistance compared to the parental strains. An evaluation of the genetic diversity of resistant and susceptible *Ae. aegypti* colonies from Switzerland indicated that there is lower diversity in the resistant colony (Bonin et al. 2008). Further investigations indicated that variations in the genes encoding cadherin and leucine aminopeptidase were responsible for the resistance. In the resistant colony, the cadherin gene encoded a nucleotide polymorphism and its expression was substantially reduced compared to that of the susceptible colony (Bonin et al. 2009).

Trypsin and chymotrypsin-like enzymes are the major digestive proteases of the larval midgut of dipteran insects and are involved in many aspects of the vector - parasite relationship (Ramalho-Ortigao et al. 2003). Moreover, they are critical enzymes in the conversion of *B. thuringiensis* protoxin to toxin. Insects with altered proteinases can evade the effects of *B. thuringiensis* toxins (Ferré & Van Rie 2002). Additionally, point mutations that provide resistance through the loss of target receptors in midgut membranes have been identified (Darboux et al. 2002). Because each point mutation required to block site-specific toxins would have to occur independently, the probability of developing resistance to *B. thuringiensis* would be proportional to the number of toxins present. The AaOGr colony remained susceptible or had a low tolerance to Cryl1Aa for at least 33 generations of selection, consistent with the observation that *Ae. aegypti* is the most susceptible mosquito species to *B. thuringiensis* toxins in the Culicidae family (Orduz et al. 1998). The trend of increasing RR values starting with the 24th generation could be evidence of slow change with regard to susceptibility towards Cryl1Aa. Understanding the mechanisms of resistance in vector species, such as *Ae. aegypti*, is key to the design of resistance management strategies and to the search for new toxins and strains of *B. thuringiensis* active against mosquitoes.

An important aspect of the mechanism of physiological adaptation and resistance by insects to *B. thuringiensis* toxins is the altered capacity to process protoxin to Cry toxin (Oppert et al. 1997). Variations in the proteolytic processing of the Cry toxin are the result of two distinct routes by which proteinases may affect toxin function, either via decreased rates of activation or increased rates of toxin degradation (Oppert et al. 1997, Bravo & Soberon 2008). In *P. interpunctella* and *Ostrinia nubilalis,* the loss of specific midgut trypsins for converting protoxin to toxin accounted for partial resistance to Cryl1A toxins (Candas et al. 2003). Resistance in other coleopterans, such as *Melolontha melolontha* and *Leptinotarsa decemlineata*, is related to this altered mechanism (Oppert et al. 1997, Karumbaiah et al. 2007). Here, zymogram assays confirmed the presence three bands between 40-70 kDa with protease activity. To verify that the proteins belonged to the serine proteases group, the gel was incubated in presence of inhibitors specific to serine proteases, resulting in the complete depletion of bands. These results suggest that serine proteases are major proteases in the midgut lumen; however the participation of trypsin and chymotrypsin in this process could not be excluded, as they cannot be examined using this approach. Specific substrates were therefore used to determine the activity of these proteases in both colonies. We examined the proteinase activity of the extracts using commercial substrates and determined the protease midgut activity at both neutral pH (7.0) and at the physiological pH (10.3) of the mosquito larvae gut because it is known that this parameter is essential to protein solubility and enzymatic activity for toxin efficacy (Segura et al. 2000, Karumbaiah et al. 2007). Using these two pH conditions, the protease activity of gut extracts was examined for larvae of both AaOG colonies after the 54th generation, when differences in the mortality kinetics were prominent. The enzymatic activity of the gut extracts was higher at pH 10.3 than at pH 7.0, consistent with the results of Segura et al. (2000). Additionally, we observed a significant decrease in trypsin and chymotrypsin activity in the resistant colony. These findings corroborate previous reports of proteinase deficiencies in other insect species that have developed resistance to *B. thuringiensis* toxins (Oppert et al. 1997, Candas et al. 2003, Karumbaiah et al. 2007), but the relative contribution of protease alterations to resistance in the AaOGr colony remains to be confirmed, particularly with regard to reports of other mechanisms of resistance. We propose to investigate the susceptibility of the AaOGr colony to other *B. thuringiensis* toxins, such as Cry4Aa, Cry4Ba, Cryl1Ba, Cryl1Bb and Cyt1A, to perform a proteomic analysis of the midgut proteins of the Cryl1Aa-susceptible and resistant colonies and to determine differences in the binding of Cryl1Aa to midgut proteins between the AaOGs and AaOGr colonies.

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