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ORIGINAL ARTICLE

Limitations of RNAi of α6 nicotinic acetylcholine receptor subunits for assessing the *in vivo* sensitivity to spinosad

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Abstract Spinosad is a widely used insecticide that exerts its toxic effect primarily through interactions with the nicotinic acetylcholine receptor. The α6 nicotinic acetylcholine receptor subunit is involved in spinosad toxicity as demonstrated by the high levels of resistance observed in strains lacking α6. RNAi was performed against the Dα6 nicotinic acetylcholine receptor subunit in *Drosophila melanogaster* using the Gal4-UAS system to examine if RNAi would yield results similar to those of Dα6 null mutants. These Dα6-deficient flies were subject to spinosad contact bioassays to evaluate the role of the Dα6 nicotinic acetylcholine receptor subunit on spinosad sensitivity. The expression of Dα6 was reduced 60%–75% as verified by quantitative polymerase chain reaction. However, there was no change in spinosad sensitivity in *D. melanogaster*. We repeated RNAi experiments in *Tribolium castaneum* using injection of dsRNA for *Tcasα6*. RNAi of *Tcasα6* did not result in changes in spinosad sensitivity, similar to results obtained with *D. melanogaster*. The lack of change in spinosad sensitivity in both *D. melanogaster* and *T. castaneum* using two routes of dsRNA administration shows that RNAi may not provide adequate conditions to study the role of nicotinic acetylcholine receptor subunits on insecticide sensitivity due to the inability to completely eliminate expression of the α6 subunit in both species. Potential causes for the lack of change in spinosad sensitivity are discussed.

Key words bioassay, insecticide target site, nicotinic acetylcholine receptor, RNAi, spinosad

Introduction

The nAChRs mediate excitatory cholinergic neurotransmission in the central nervous system of insects. The nAChRs are members of the Cys-loop ligand-gated ion channel superfamily, which includes receptors for serotonin, glycine and GABA (Ortells & Lunt, 1995). The nAChR contains three functional domains: an extracellular *N*-terminal ligand-binding domain; four transmembrane segments (TM1–4), of which TM2 forms the pore of the receptor channel; and the intracellular linker between TM3–4, which is responsible for receptor desensitization and intracellular trafficking (Imoto *et al.*, 1988; Corringer *et al.*, 1995; Millar & Harkness, 2008). Native nAChRs of insects are homopentamers of α subunits, or heteropentamers of α and β subunits (Sattelle *et al.*, 2005; Millar, 2009; Millar & Denholm, 2007). The α subunits possess a YxCC motif in loop C of the ligand-binding domain, whereas β subunits do not. Insects have between 10 and 16 nAChR genes (Sattelle *et al.*, 2005; Jones & Sattelle, 2007; Shao *et al.*, 2007; Jones *et al.*, 2010), and can generate tremendous diversity of nAChR subunit proteins through posttranscriptional modifications such as alternative splicing and A-to-I RNA editing. For

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example, alternative and cassette exon use in *Tcasα6* from *Tribolium castaneum* can generate more than 256 splicing isoforms (Rinkevich & Scott, 2009), and more than 30 000 unique transcripts are possible from *Dα6* of *Drosophila melanogaster* using combinations of alternative splicing and A-to-I RNA editing (Grauso *et al.*, 2002). The posttranscriptional modifications of insect α6 subunit orthologs are evolutionarily conserved (Tian *et al.*, 2008; Jin *et al.*, 2007). The nAChRs are the target for two major groups of insecticides: spinosyns (such as spinosad) and neonicotinoids (such as imidacloprid). However, the role of individual nAChRs (and their variously spliced and edited forms) in modulating responses to insecticides are not well known.

Spinosyns, such as spinosad, are a widely used and economically important class of insecticides. Spinosad has been used to successfully control many agricultural, domestic, and public health pests (Salgado & Sparks, 2005; Stough *et al.*, 2009). This wide spectrum of effectiveness, combined with reduced impacts on nontarget insects and the environment and low vertebrate toxicity are all desirable and beneficial aspects of spinosad (Thompson *et al.*, 2000; Salgado & Sparks, 2005). Spinosad exerts its toxic effects through interactions with the nicotinic acetylcholine receptor by acting as an allosteric modulator (Salgado & Sparks, 2005). Through a series of mutagenesis experiments, it has been shown that *D. melanogaster* strains lacking Dα6 are 370–1100-fold resistant to spinosad (Perry *et al.*, 2007; Watson *et al.*, 2010). In addition, spinosad resistance in diamondback moth is due to errant splicing that introduces premature stop codons in *Pxylα6* (Rinkevich *et al.*, 2010). These results clearly indicate a role for *α6* subunits in the mode of action of spinosad.

RNAi has been used in numerous biological fields, including insecticide toxicology (Rajagopal *et al.*, 2002; Lycett *et al.*, 2006; Bautista *et al.*, 2009). However, the use of RNAi to test the role of different nAChRs in insecticide mode of action has not been reported. If RNAi could be used to effectively silence one or more nAChR subunits, this would allow for new studies that could elucidate the relative importance of each receptor to insecticide toxicology. As a starting point, we used RNAi of α6 in two species of insect (*D. melanogaster* and *T. castaneum*) to examine the effect on toxicity to spinosad, given that α6 null strains are 370–1100-fold resistant to spinosad. While the levels of α6 transcripts could be reduced using Gal4-driven expression of dsRNA (*D. melanogaster*) or dsRNA injection (*T. castaneum*), the toxicity to spinosad did not change. Possible reasons for these results are discussed.

Materials and methods

Insects

Six strains of *D. melanogaster* were used in this study. The actin and elav strains expressed Gal4 ubiquitously and in the nervous system, respectively, and were balanced over CyO. The Dα6RNAi strain expressed dsRNA under the control of UAS. The Df(2L)s1402 and Df(2L)Exel6025 strains had chromosomal deletions that lacked the Dα6 locus. The w^{1118} strain is a partial deletion of the *white* gene. The actin (stock #25374), Dα6RNAi (#25835), Df(2L)s1402 (#556), Df(2L)Exel6025 (#7508), and w^{1118} (#3605) strains were obtained from the Bloomington Stock Center. The elav strain was kindly provided by Hanna Kim (Cornell University, Department Neurobiology and Behavior).

Reciprocal crosses of flies expressing Gal4 under specific promoters and Dα6RNAi were performed to activate the expression of the dsRNA (i.e., actin♀ × Dα6RNAi♂ and Dα6RNAi♀ × actin♂). The offspring that had the CyO marker were discarded so that only flies expressing dsRNA for Dα6 were used in bioassays. Flies were reared on cornmeal-agar-based media supplemented with dextrose and held at 25°C, 30% RH, and 12 : 12 light–dark photoperiod. Flies were transferred to new vials weekly.

One strain of red flour beetles (*T. castaneum*) was used in this study. The wild-type, insecticide-susceptible GA-1 strain was obtained from Kathy Leonard at Kansas State University and reared, as previously described (Rinkevich & Scott, 2009).

RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction of Dα6

Total RNA was isolated from 5, unsexed, 1–5 day old *D. melanogaster* adults using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Total RNA was resuspended in 100 μL of diethylpyrocarbonate (DEPC)-treated H_2O. RNA concentration was measured on a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE). Reverse transcription was performed with 5 μg of total RNA using Go-Script (Promega, Madison, WI) according to the manufacturer's directions.

Expression of Dα6 relative to actin5c was evaluated in triplicate for each sample using 20 μL Power SYBER (Applied Biosystems, Carlsbad, CA) with 25 ng of cDNA on a CFX96 real-time polymerase chain reaction (PCR) detection system (Bio-Rad, Hercules, CA). The primer set RTActinF and RTActinR was used to measure actin5c

Table 1 Sequences of primers used.

Primer	Sequence
T7Tcasa6F0	TAATACGACTCACTATAGGGTCTTACAATGGCTGCCGTGGATG
T7Tcasa6R1	TAATACGACTCACTATAGGGTCGCAAATTCGCAAAATCGCAGA
Tcasa6F1	CACGAAAAGCGGCTACTAAACA
Tcasa6R1	GTTTGTTTGGAAAGTCCCGTCGA
RTActinF	CCCAAGGCCAACCGTGAGAAGATG
RTActinR	GACCGGAGGCGTACAGCGAGAGC
RTDa6F	CGTCGCTGTCGCTGTTTGTCC
RTDa6R	CAGGGGCTCCGATTCATTGG

expression and the primer set RTDa6F and RTDa6R was used to measure Dα6 expression (Table 1). Three independent batches of cDNA from each cross and parental strain were used. The thermocycler program was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 58°C for 1 min. Fluorescence was measured at the end of each cycle. Relative expression of Dα6 was evaluated using the $2^{-\Delta\Delta Ct}$ method and statistically compared using Student's *t*-test.

RNA extraction, cDNA synthesis, and in vitro *production of* Tcasα6 *dsRNA*

RNA isolation and cDNA synthesis were performed on 5, unsexed, 1–5 day old *D. melanogaster* adults, as described above. A 723 bp fragment of *Tcasα6*, corresponding to base 1 025 of the open reading frame through base 251 of the 3′ untranslated region of the mRNA, was amplified by PCR in 100 μL Go-Taq (Promega, Madison, WI) reactions using the primers T7Tcasa6F0 and T7Tcasa6R1 (Table 1). The PCR conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1.5 min with a final extension step at 72°C for 10 min. The PCR product was purified by QIAEXII gel purification kit (Qiagen, Valencia, CA) after electrophoresis on 1% agarose gel. The purified product was used as DNA template for dsRNA synthesis.

Synthesis of dsRNA was performed using the AmpliScribe T7-Flash transcription kit (Epicentre, Madison, WI) according to the manufacturer's instructions. The transcribed product was DNase I treated, then phenol/chloroform (1:1) and chloroform extracted, respectively, and precipitated with ammonium acetate, followed by a 70% ethanol wash. The dsRNA pellet was dissolved in distilled H_2O at a concentration of 3 μg/μL and stored at −70°C until use.

One-day old *T. castaneum* pupae were dorsally fixed to double-sided tape on a glass slide and injected with 600 ng of dsRNA for *Tcasα6* (in a 0.22 μL volume) between the second and third ventral abdominal segments using a Picospritzer II pressure system (General Valve Corp, Fairfield, NJ). The same amount of distilled H_2O was injected for control. Uninjected beetles were used as an injection control. The injected pupae were placed into wheat flour fortified with 5% (wt/wt) yeast after 1 h incubation at room temperature and kept in continuous darkness at 27°C and about 65% RH for 1 week until they emerged as adults to be used in bioassays.

RNA extraction was performed as described above on control injected and *Tcasα6* dsRNA injected beetles 1 week after injection. Reverse transcriptase PCR for *Tcasα6* was conducted using 1 μg of RNA in 25 μL one-step PCR reactions (Qiagen, Valencia, CA) according to the manufacturer's directions with the primers Tcasa6F1 and Tcasa6R1 (Table 1). The PCR conditions were as follows: 50°C for 60 min, 94°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec with a final extension step at 72°C for 10 min. The PCR was visualized on a 1% agarose gel.

Spinosad bioassays

Spinosad (96%, Elanco Animal Health, Indianapolis, IN) was dissolved in acetone to create 20 mg/mL stock solutions from which the appropriate serial dilutions were made. Contact bioassays were performed by treating a scintillation vial (Wheaton, Millville, NJ; internal surface area = 38.6 cm^2) with 0.5 mL of insecticide solution. Controls vials were treated with 0.5 mL of acetone only. Vials were placed in a fume hood and rolled to evenly coat the inner surface of the vials. Treated vials were allowed to dry for 1 h. Twenty, unsexed, 1–5 day old adult

Table 2 Toxicity of spinosad and Dα6 expression in strains and crosses of *D. melanogaster*.

Strain or cross	$n^{\dagger}$	Spinosad LC_{50} (ng/cm^2)‡	Slope§	Relative Dα6 expression
w^{1118}	560	4.9 (3.9–6.0)	1.9 (± 0.2)	NA
Df(2L)s1402	630	6 100 (2 190–9 270)	0.9 (± 0.1)	NA
Df(2L)Exel6025	646	36 300 (22 000–63 500)	0.6 (± 0.1)	NA
actin	1 100	25.0 (22.4–28.0)	2.4 (± 0.2)	NA
elav	672	15.1 (12.0–18.5)	1.3 (± 0.1)	NA
Dα6RNAi	431	63.1 (52.0–79.6)	2.4 (± 0.2)	1.00 (0.07)
actin × Dα6RNAi	262	57.9 (31.5–90.7)	1.2 (± 0.2)	0.44* (0.05)
Dα6RNAi x actin	250	84.1 (39.0–146.0)	0.9 (± 0.1)	0.40* (0.04)
elav × Dα6RNAi	385	75.5 (60.5–87.0)	3.2 (± 0.6)	0.44* (0.16)
Dα6RNAi × elav	930	74.6 (64.6–83.3)	2.7 (± 0.3)	0.25* (0.02)

†Number of animals tested.

‡Numbers in parenthesis represent the 95% confidence interval of the LC_{50}.

§Number in parenthesis is the standard error.

*Values are significantly different from 1.0. The number in parenthesis is the standard deviation.

NA: not assessed.

flies or beetles were placed in a vial covered with white nylon tulle and plugged with a cotton ball. The cotton balls were wetted daily with 10% sucrose water. Bioassays were conducted at the rearing conditions used for each insect as described above. A minimum of three replicates for each concentration that gave more than 0% and less than 100% mortality were performed. Mortality was assessed at 72 h posttreatment.

Bioassay data were pooled for each replicate and analyzed by standard Probit analysis as adapted for personal computer use (Raymond, 1985) using Abbott's correction for control mortality (Abbott, 1925). The LC_{50} values of each strain, cross (flies) and dsRNA treatment (beetles) were considered to be significantly different if the 95% confidence intervals did not overlap.

Results

Drosophila

The Gal4-UAS system significantly reduced the expression of Dα6 to 25%–44% relative to the parental Dα6RNAi strain using actin and elav crosses (Student's *t*-test, $P < 0.05$, Table 2). The spinosad LC_{50} values of each cross were significantly higher than the parental driver strains (two- to five-fold), but there were no significant differences in the LC_{50} values compared to the Dα6RNAi parental strain (Table 2). Bioassays with the Dα6 deficient strains Df(2L)s1402 and Df(2L)Exel6025 showed very high levels of spinosad resistance (1244 and 7408 fold, respectively) compared to the w^{1118} strain from which they were derived (Table 2), which agrees with previous work (Perry *et al.*, 2007; Watson *et al.*, 2010). Therefore, although Dα6 null strains are highly resistant to spinosad, reduced expression of Dα6 by RNAi is insufficient to significantly alter spinosad sensitivity.

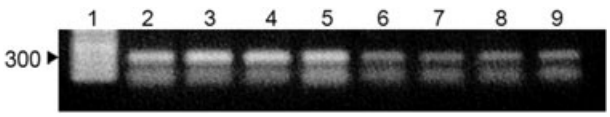

Fig. 1 Semiquantitative PCR analysis of *Tcasα6* expression in *T. castaneum* demonstrates a nearly 50% reduction in *Tcasα6* via *Tcasα6* dsRNA injection. Lane 1 is 5 μL of 1 kb Plus ladder (Invitrogen, Carlsbad, CA), lanes 2–5 are bands from control injected beetles, and lanes 6–9 are from beetles injected with *Tcasα6* dsRNA. The number to the left of lane 1 indicates the size of the band in lane 1 in bp. Gel image courtesy of Dr. J.-R. Gao.

Tribolium

Based on semiquantitative real-time PCR, *Tcasα6* dsRNA injection reduced *Tcasα6* expression by nearly half (Fig. 1). Nevertheless, there were no significant differences in the spinosad LC_{50} values between control ($LC_{50} = 618$ ng/cm^2 [95% CI 446—891]), sham injected ($LC_{50} = 130$ ng/cm^2 [95% CI 85–497]), and *Tcasα6* dsRNA injected beetles ($LC_{50} = 444$ ng/cm^2 [95% CI 203–1056]). These results mirror the lack of change in spinosad insensitivity despite reduction in α6 expression as seen in *D. melanogaster* (Table 2). Lower amounts of

dsRNA were also tested, but these produced similar results (or smaller reductions in silencing, data not shown).

Discussion

Based on our results, it appears that RNAi of nAChRs has limited utility for studies of spinosad, and perhaps other insecticides working at this target site. There are a few reasons why this may be the case: recessive pattern of inheritance, protein stability, difficulties in dsRNA uptake in the nervous system, or intrinsic properties of the species, gene, or tissue. Spinosad resistance in α6 null strains is highly recessive (Perry *et al.*, 2007; Rinkevich *et al.*, 2010), and insecticides may only need to affect a small number of target sites to exert their toxic effects (Tatebayashi & Narahashi, 1994). Thus, the inability of RNAi to completely abolish expression of Dα6 may explain the ineffectiveness of RNAi on the LC_{50} of spinosad in our experiments.

It is possible that the rate of protein turnover for nAChRs is an important factor for the effectiveness of RNAi, and nAChRs can be stable for more than 2 weeks (Lomazzo *et al.*, 2011). Whether or not nAChR protein stability contributed to our seeing no change in spinosad susceptibility is difficult to determine because transcripts were still detected in strains subjected to our RNAi protocols.

There may be difficulties with dsRNA uptake and RNAi effectiveness in specific tissues. For example, larval injection of dsRNA to assess RNAi in the salivary glands of *Anopheles gambiae* requires 10-fold higher amounts of dsRNA to reduce gene expression to similar levels in the midgut and ovary (Boisson *et al.*, 2006). However, in our experiments, the 600 ng of *Tcasα6* dsRNA injected should have been adequate to induce RNAi and effectively reduce expression of *Tcasα6* because minute amounts dsRNA can cause a shift in phenotype in *T. castaneum* larvae (Tomoyasu & Denell, 2004).

The effectiveness of RNAi may be an intrinsic feature of the species, gene, or tissue (Belles, 2010). RNAi has been effectively used in *D. melanogaster* and *T. castaneum*, so species specific factors, such as dsRNA degradation efficiency, spread of RNAi signaling, and muted response of RNAi machinery, are not a cause of the ineffectiveness of RNAi in our experiments. Properties of the gene may be involved with the ineffectiveness of RNAi for the α6 subunit, but these prospects are speculative at best. Gene specific features of RNAi insensitivity include α6 dsRNA degradation, increased transcription rate of α6 subunits upon dsRNA administration, and protection from RNases. The likely cause of the ineffectiveness of RNAi for α6 subunits is dependent on tissue specific factors, such as the

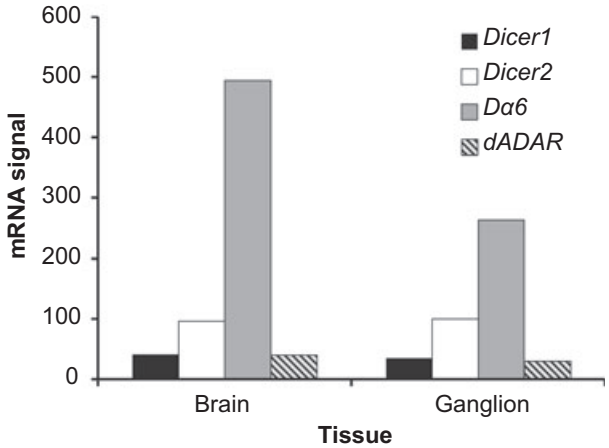

Fig. 2 Expression levels of *Dicer1*, *Dicer2*, *Dα6*, and *dADAR* in the brain and thoracicoabdominal ganglion in *D. melanogaster.* Data in this figure was obtained from FlyAtlas (Chintapalli *et al.*, 2007).

observation that RNAi machinery is not highly expressed in the nervous system. In *D. melanogaster*, the mRNA abundance for both *Dicer1* and *Dicer2* in the adult brain and thoracicoabdominal ganglion is much lower than *Dα6* expression in those tissues (Fig. 2, Chintapalli *et al.*, 2007). The disparity in expression of these genes in these tissues may explain the ineffectiveness of Dα6 RNAi in evaluating spinosad sensitivity. Another study has shown that RNAi of nAChRs is more difficult than other genes. In the brown planthopper, *Nilaparvata lugens*, injection of dsRNA for globally expressed calreticulin and the gut specific cathepsin-B decreased expression of those genes by 40%, but injection of dsRNA for *Nlβ2*, an nAChR subunit, only reduced expression by 25% (Liu *et al.*, 2010). The effect of *Nlβ2* RNAi on insecticide sensitivity has not been evaluated.

Difficulties of tissue penetration in the nervous system are likely not the cause of ineffective RNAi, because RNAi of *dADAR*, which is responsible for A-to-I RNA editing in the nervous system in *D. melanogaster*, is very effective and informative (Jepson & Reenan, 2009). The expression levels *dADAR*, *Dicer1*, and *Dicer2* in the brain and thoracicoabdominal ganglion are similar (Fig. 2) (Chintapalli *et al.*, 2007); thus supporting the hypothesis that large differences in expression levels of RNAi target genes, compared to *Dicer1* and *Dicer2,* may be responsible for the incomplete suppression of α6 in our experiments.

Based on the above, it appears that the most likely explanations for why spinosad resistance was not observed in our α6 RNAi experiments was that complete suppression of the α6 subunit may be required to

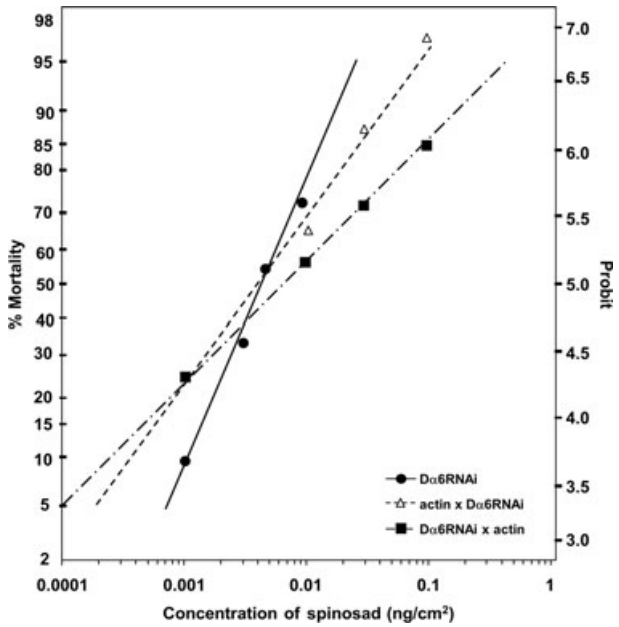

Fig. 3 Log concentration-probit graph of spinosad toxicity to three lines of *D. melanogaster*. Concentrations that gave 0 or 100% mortality are not shown.

generate spinosad resistance, but only 60%–75% reduction in α6 transcripts was achieved using RNAi. A possible explanation for the incomplete suppression of α6, particularly in *D. melanogaster*, was due to the difference in the expression levels of *Dα6* (high) and *Dicer1* and *Dicer2* (low). This hypothesis is supported by other reports that demonstrate the lack of complete silencing of other nAChR subunits (Vermehren & Trimmer, 2005; Liu *et al.*, 2010).

Our bioassays also reinforce the potential problem of evaluating the effectiveness of RNAi using a single concentration or dose of insecticide (Rajagopal *et al.*, 2002; Lycett *et al.*, 2006; Revuelta *et al.*, 2009; Zhang *et al.*, 2010; Revuelta *et al.*, 2011). Results from using a single concentration may be misleading because they assume the slopes of the concentration response curves between test populations are equal. A one-concentration survivorship test would result in Type 1 error because the slopes varied between treatments (Table 2). For example, the LC_{50} values for Dα6RNAi, actin × Dα6RNAi and Dα6RNAi × actin were not significantly different (Table 2). However, if we used the LC_{95} of spinosad for the Dα6RNAi strain, the actin × Dα6RNAi and Dα6RNAi × actin would have only shown 75% and 66% mortality, respectively, giving the impression that ubiquitous reduction in Dα6 expression decreases spinosad sensitivity (Fig. 3). Conversely, if the reciprocal crosses of actin and Dα6RNAi were tested at the LC_5 of spinosad to the parental Dα6RNAi, the higher mortality of the cross-offspring (~24%) would suggest that ubiquitous reduction of Dα6 increases spinosad sensitivity (Fig. 3). This demonstrates that the dose or concentration applied to test for insecticide sensitivity may yield contradictory and inaccurate assessments of the effect of RNAi on insecticide sensitivity, and all future experiments in this field should be considerate of this fundamental aspect of toxicology.

RNAi has been effectively used to study critical processes such as embryogenesis, differentiation, digestion, and sex determination in diverse insects such as *Drosophila*, *Tribolium*, and *Bombyx* (Meihls *et al.*, 2008; Hossain *et al.*, 2008; Parthasarathy & Palli, 2009). RNAi in *Tribolium* may require as little as 5 pg of dsRNA to produced a noticeable phenotypic change and the reduced systemic expression of genes via injection of dsRNA can last up to 2 months (Tomoyasu & Denell, 2004). Recent developments have explored the use of RNAi as a form of agricultural pest control by administering dsRNA as a spray or in transgenic plants (Huvenne & Smagghe, 2010). While these applications of RNAi have yielded valuable insights to the function of many genes, we were not able to produce significant phenotypic changes in spinosad sensitivity by using RNAi for the same target using two methods in two different insects. Our results suggest RNAi against nAChRs may not be an appropriate method to study the role of individual nAChRs in insecticide toxicology.

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Disclosure

The authors have no conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject of this manuscript.

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