



Mortality and Cholinesterase Inhibition in Butterflies Following Aerial Naled Applications for Mosquito Control on the National Key Deer Refuge

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Abstract

Natural resource managers are concerned about the impacts of aerial ultra-low volume spray (ULV) of insecticides for mosquito control (i.e., mosquito adulticides) and seek science-driven management recommendations that reduce risk but allow vector control for nearby human populations. Managers at the National Key Deer Refuge (Florida Keys, FL) are concerned for ULV effects upon conservation efforts for imperiled butterflies (Florida leafwing [*Anaea troglodyta florida-lis*] and Bartram's hairstreak [*Strymon acis bartrami*] butterflies). No-spray zones were designated for protection of those butterflies, but their effectiveness for mitigation is unclear. To address this uncertainty, cholinesterase activity (ChE) and mortality were monitored for caged butterflies gulf fritillary [*Agraulis vanilla*] and great southern white [*Ascia monuste*] deployed on the Refuge during three aerial ULV applications of the insecticide naled. Residue samplers also were deployed to estimate butterfly exposure. Spray efficacy against mosquitoes was assessed by deploying caged mosquitoes at the same locations as the butterflies. Average naled residue levels on filter paper samplers in the target area (1882–2898 $\mu\text{g}/\text{m}^2$) was significantly greater than in the no-spray zone (9–1562 $\mu\text{g}/\text{m}^2$). Differences between the no-spray zone and target area for butterfly mortality and ChE were inconsistent. Average mortality was significantly lower, and average ChE was significantly higher in the no-spray zone for larvae of one species but not for larvae of the other species. Mosquito mortality did not differ significantly between the two areas. Data from the present study reflect the inconsistent effectiveness of no-spray zones on the Refuge using standard methods employed at the time by the vector control agency in the Florida Keys and possibly by other vector control agencies in similar coastal environments. Furthermore, these findings helped to guide the design and to improve the conservation value of future no-spray zone delineations while allowing for treatment in areas where mosquito control is necessary for vector-borne disease reduction.

Conservation of imperiled butterfly species has been a difficult resource management issue for resource managers. The National Key Deer Refuge (Refuge) is in a challenging landscape for conservation of rare invertebrates due to its landscape that is fragmented among residential neighborhoods, refuge lands, and coastal saltmarsh and mangrove

habitats. That landscape complicates management actions such as controlled burns for maintenance of habitat conducive for two resident endangered butterfly species. Located on Big Pine Key in the Florida Keys, the Refuge is home to several imperiled species, including Bartram's hairstreak butterfly (*Strymon acis bartrami*), and historically the Florida leafwing butterfly (*Anaea troglodyta florida-lis*). In 2014, both butterfly species were listed as endangered under the Endangered Species Act, which resulted in the designation of significant portions of Refuge land as critical habitat for protection of both butterfly species (USFWS 2014). In addition to the imperiled species, very high densities of mosquitoes are found within the Refuge due to the presence of saltmarsh and mangrove wetland habitats. Those mosquitoes are potential disease vectors that present a health threat for residents of adjacent communities with which the Refuge is highly integrated. As a result, the Refuge has permitted

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limited pesticide applications on its property to control mosquitoes (USFWS 2014). Given that some pesticides used for mosquito control may also affect nontarget organisms, including butterflies, permitting mosquito control activities on the Refuge impacts conservation efforts.

Three primary mosquito control methods are practiced within and adjacent to the Refuge: larviciding with *Bacillus thuringiensis israelensis*, ground-based (i.e., from trucks) ultra-low volume (ULV) sprays of pesticides containing the active ingredient permethrin, and aerial ULV sprays of pesticides containing the active ingredient naled. Larviciding and aerial ULV sprays of naled are permitted on Refuge but ground based ULV sprays of permethrin are not. Aerial ULV applications typically cover the entirety of Big Pine Key, including the Refuge, except for designated no-spray zones that encompass habitat within the Refuge considered critical for conservation of imperiled species.

Naled is an organophosphate (OP) insecticide that is the active ingredient in pesticide products used for aerial ULV spraying in the Florida Keys. Naled is toxic to butterflies (Hoang et al. 2011; Bargar 2012a; USFWS 2014) and has been categorized by the USEPA as highly toxic to terrestrial invertebrates (USEPA 2006). Naled also has low environmental stability being very susceptible to photolysis and hydrolysis resulting in short environmental half-lives of 1.3–8.2 h depending on humidity and light levels (Tietze et al. 1996; USEPA 2006). Based on acute toxicity to honey bees, application rates, and modeled environmental fate characteristics for naled, the USEPA stated that “endangered terrestrial invertebrates would be at risk from all uses of naled” (USEPA 2006). In fact, elevated risk for butterflies as a result of naled applications has been suggested (Salvato 2001; Zhong et al. 2010; Hoang et al. 2011; Bargar 2012b). As a result, naled applications on the Refuge is a concern for resource managers attempting to conserve the two imperiled butterfly species.

Cholinesterase (ChE) is an enzyme critical for proper nerve function in vertebrate and invertebrate organisms. It hydrolyzes the neurotransmitter acetylcholine, making the post-synaptic receptor to which acetylcholine was bound available for the next neurotransmission (Fukuto 1990). Because of its structural similarity to acetylcholine, OP insecticides like naled also bind ChE. But, unlike acetylcholine, the binding of OP insecticides with ChE is less reversible meaning OP insecticides reduce the amount of ChE available in the neurosynapse to hydrolyze acetylcholine (Colovic et al. 2013). In the absence of ChE, acetylcholine levels remain elevated in the neurosynapse causing continuous neurostimulation, which can alter behavior (Brewer et al. 2001; Cooper and Bidwell 2006) and lead to death (Coppage and Matthews 1975; Ludke et al. 1975). As a result, the activity of ChE in blood plasma (Goldstein et al. 1999; Maul and Farris 2005; Fildes et al. 2006; Martinez-Haro

et al. 2007) or the brain (Kumar and Chapman 1998; Morgan et al. 1990; Varo et al. 2008) is used as a biomarker of exposure to OP pesticides.

The primary objective for the present study was evaluation of the no-spray zone’s effectiveness at reducing risk for butterflies in that zone. Commercially available surrogate butterflies (adults and larvae) were deployed in exposure chambers in areas of the Refuge targeted by aerial applications (target area) and in an adjacent area not targeted by the applications (nontarget area) to measure their response following aerial applications. Cholinesterase activity was chosen as the primary indicator of exposure, because it is responsive to OP pesticide exposure (Mazur and Bodansky 1946; Weiss 1961; Day and Scott 1990). In addition to the butterflies, mosquitoes were deployed at the same locations in separate exposure chambers to determine whether naled exposure was adequate for mosquito mortality. Lastly, residue samplers were deployed to provide an indication of relative naled exposure among the locations.

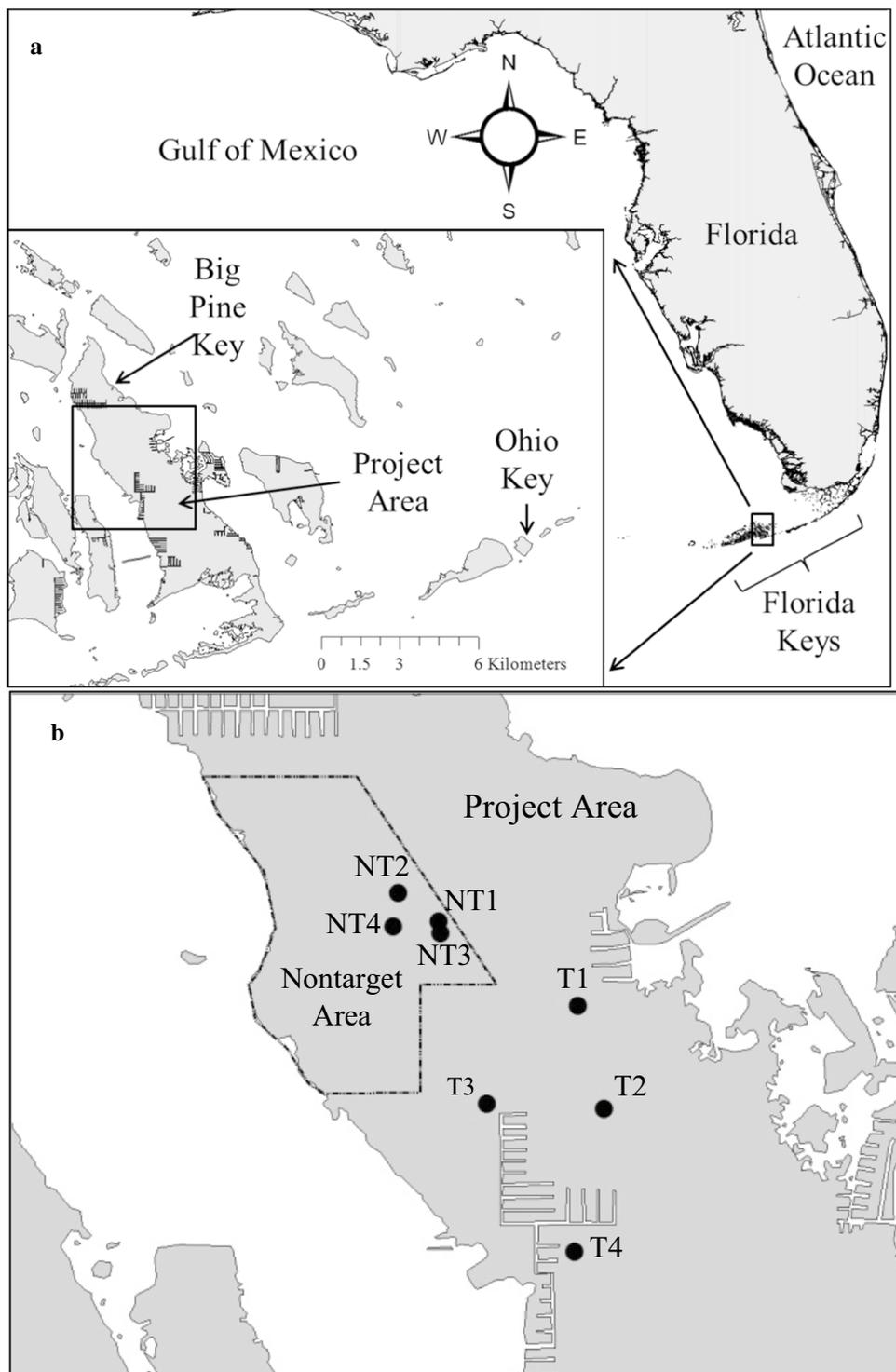
Methods

Project and Sampling Locations

The present study occurred within the Refuge, which includes a significant portion of Big Pine Key in the Florida Keys (Fig. 1). Pine rockland and hardwood hammock are the primary upland habitats on Big Pine Key. The pine rockland habitat is characterized by slash pine stands with an open canopy and a low-profile palm understory. It is a fire-maintained community that transitions to hardwood hammock in the absence of periodic fires and also is the habitat critical for pineland croton, which is the host plant for the Bartram’s hairstreak and Florida leafwing butterflies. The present study was conducted on the Refuge under a special use permit issued by the Refuge.

All chosen locations for the present study were in the pine rockland habitat. Nine different locations within the Refuge were chosen for the present study. Four locations in a no-spray zone, herein after referred to as the nontarget area, were chosen to evaluate response due to drift, while four locations in an area targeted by the aerial applications, herein after referred to as the target area, were chosen to evaluate response from intentional exposure. A single reference location was selected where no aerial applications were expected during the respective field trial. The reference location during Field Trial 1 was in a nontarget area (Cactus Hammock) on the southern end of Big Pine Key. However, based on observations during that field trial, the reference location may have been exposed to naled due to drift from the adjacent target area. Therefore, the reference

Fig. 1 Location of the project area on Big Pine Key in the Florida Keys, Florida, USA (a); and locations in the target area (TA) and nontarget area (NTA) at which caged organisms and residue samplers were placed (b)



location for Field Trials 2 and 3 was moved to Ohio Key (Fig. 1).

Applications

Field trials took place during aerial applications conducted by the Florida Keys Mosquito Control District in response

to elevated mosquito populations on Big Pine Key and in accordance with the Pesticide Special Use Permit issued to the Refuge by the U.S. Fish and Wildlife Service. Aerial application of Trumpet EC (AMVAC, Los Angeles, CA) occurred at a rate of 70.9 g of active ingredient naled per hectare from an altitude of 30.5 m over the Refuge. The plane (Britten-Norman Islander BN-2T) was equipped

with a Micronair AU4000 atomizer (Micron Sprayers Ltd, Herefordshire, UK), a Wingman GX aerial spray manager (Adapco Inc., Sanford, FL), and an AIMMS weather monitor (Aventech Research, Inc, Barrie, ON, CA). Three separate applications occurred: June 10 and July 22 of 2009 (Field Trials 1 and 2, respectively), and the third on June 15 of 2011 (Field Trial 3). The atmospheric conditions at the time of each field trial are shown in Table 1.

Test Organisms

The surrogate butterfly species utilized in this study were the gulf fritillary (*Agraulis vanillae*) and great southern white (*Ascia monuste*) butterflies. Both are native to south Florida, and one (gulf fritillary) is in the same family (Nymphalidae) as the imperiled Florida leafwing butterfly. The surrogate butterfly species were obtained from a commercial vendor (Shady Oak Butterfly Farm, Brooker, FL). Genetic diversity of the vendor's stock is maintained through biannual introduction (10–20% of total stock) of wild-caught butterflies. Before shipment to the Refuge, adult butterflies were placed into glassine envelopes and packed within an insulated box with cold packs. Larvae (4–5th instar) were placed into plastic bags with host plant and placed into an insulated box with cold packs. All adults and larvae were shipped priority overnight to ensure their receipt on the morning of the day before the field trial. Survival during transit was >90% for adult gulf fritillary butterflies as well as for the larvae. Survival during transit was lower for adult great southern white butterflies (80% for Field Trial 1 and lower for Field Trial 2) indicating lower handling tolerance for that species. Because of the handling hypersensitivity of this species, it was not used during Field Trial 3. Upon receipt at the Refuge, adults and larvae were randomly assigned into nine groups (4 target area locations, 4 nontarget area locations, and 1 reference location) for placement into the exposure chambers. Multiple species were combined within a single exposure chamber for adults, because their food requirement is nonspecific, whereas larvae of different species were placed into separate chambers because of specific food requirements. The number of adults and larvae placed into each exposure chamber varied depending on availability. For adult butterflies, the number per chamber ranged from 11 to 14 for great southern

white and 4–11 for gulf fritillary butterflies. For larvae, the number ranged from 6 to 15 for great southern white and 6–17 for gulf fritillary. Larval food (host plant leaves) was added to the exposure chambers, but only before and after the deployments to minimize exposure via ingestion. No food was provided for adults during Field Trial 1. However, food was added (fruit or sugar solution-soaked paper towels) before and after deployments during Field Trials 2 and 3 to minimize the effect of starvation on survival.

Mosquitoes used for the field trials were wild adult female salt marsh mosquitos (*Aedes taeniorhynchus*) trapped (CO₂ traps) on the day before the trial. Captured mosquitoes were divided among exposure chambers (approximately 50 per cage) and placed into a darkened cooler before deployment. Wet paper towels were placed into the cooler to maintain humidity while cotton balls soaked with 10% sugar solution were placed on top of each screen cage to maintain mosquito vitality (Coluzzi 1964).

Exposure Chambers

The exposure chambers for adult butterflies were constructed of woven shade cloth (shading factor ~30%) and a pair of plastic embroidery hoops. The resulting cage was a cylinder with dimensions of approximately 46-cm tall × 25-cm wide (Fig. 2). Those exposure chambers were suspended from a polyvinylchloride (PVC) tripod in the field such that the bottom of the cage was approximately 60 cm above the ground. Exposure chambers for larvae during the first two field trials were plastic cups (12-cm tall × 9-cm wide), and then small mesh cages for Field Trial 3. A layer of Vaseline was added to the inner rim of the plastic cups to prevent larvae from escaping. Mosquito exposure chambers were small (12-cm tall × 4-cm wide) wire screen cages.

Residue Samplers

Two types of samplers were used to capture naled residues during the field trials as described in Zhong et al. (2010). The first was a glass fiber filter paper (Whatman No. 4 qualitative, cat no. 1004-240, surface area of 452.4 cm²) affixed to an aluminum foil-covered Styrofoam block laid flat on the ground. The second was a ~5.79 m length of acrylic yarn

Table 1 Atmospheric conditions at the time of each aerial naled application

Field trial	Date	Sunrise (h) ^a	Spray start (h)	Spray end (h)	Temperature (°C)	% Relative humidity	Wind speed (kph)
1	10-Jun-09	0635	0619	0729	26.1	76	14.5
2	22-Jul-09	0649	0628	0742	27.8	85	14.5
3	15-Jun-11	0636	0630	0758	28.3	70	11.3

^aBig Pine Key, Florida, USA



Fig. 2 Photograph of the deployment cage used for exposing surrogate adult butterflies during the field trials

strung within a vertical PVC frame. The frame was affixed to a PVC stand to allow placement on the hard, bedrock-outcropped substrate typical of the Refuge. The yarn samplers were at the same elevation as the butterfly exposure chambers. Three filter paper samplers and three yarn samplers were placed at each location in an arrangement that encircled the exposure chambers.

Field Exposures

All exposure chambers and residue samplers were deployed at their respective locations no earlier than 1 h before aerial applications. One chamber with adult butterflies and two chambers with larvae (1 for each species) were placed at each location. One cage of mosquitoes was placed at each target and nontarget area location, whereas four were placed at the reference location. At the time of application, 200 μL of a solution with a known naled concentration (2.5 mg/mL) was pipetted onto two of the three yarn and filter paper

samplers at the reference location for determination of naled recovery during sampler retrieval, extraction, and analysis.

Retrieval of all residue samplers and exposure chambers began approximately 1 h after the application. Yarn and filter paper samplers were placed into separate foil-wrapped, 40-mL clear glass or amber glass vials. One set of precleaned forceps was used to collect the yarn while a second set was used to collect the filter papers. The forceps were cleaned after use at each location by wiping them with alcohol-soaked wipes. The vials were transported back to the Refuge headquarters, filled with 30 mL of the extraction solvent (pesticide-grade hexane), and kept in a freezer ($-10\text{ }^{\circ}\text{C}$) until transport to the laboratory. All butterflies remained in their exposure chambers during transport back to the Refuge headquarters and were monitored for 24 h following the aerial application. Any dead adults found during the 24-h monitoring period were placed into separate labeled glassine envelopes. Adults alive at the end of the 24-h monitoring period were placed into separate labeled glassine envelopes, whereas larvae were placed separately into labeled vials. All larvae and adults were placed into a freezer ($-10\text{ }^{\circ}\text{C}$) at the Refuge, and then into a cooler containing dry ice for transport back to the laboratory. Once at the laboratory, they were transferred into an ultra-cold freezer ($-80\text{ }^{\circ}\text{C}$) until enzyme analyses. Mosquitoes were aspirated into “clean cages” after transport to the Refuge headquarters and maintained as described earlier in coolers for 24 h. Mosquito mortality was noted at 4, 8, 12, and 24 h post application. Mosquito mortality data reported in this paper was at 24 h post application.

Residue Sampler Extraction

Naled residues on samplers retrieved during the first two field trials were extracted and analyzed as described in Zhong et al. (2010). The lower quantitation limits for this method were 0.6 $\mu\text{g}/\text{yarn}$ (yarn length $\sim 5.79\text{ m}$) and 13.2 $\mu\text{g}/\text{m}^2$ in Field Trial 1, and 3 $\mu\text{g}/\text{yarn}$ and 66.3 $\mu\text{g}/\text{m}^2$ in Field Trial 2. Naled residues on samplers retrieved during Field Trial 3 were extracted by sonication in three consecutive volumes (10 mL each) of hexane. Those were combined and concentrated under high-purity nitrogen gas to 1.5 mL, exchanged to methanol, and concentrated to a final volume of 1 mL. The detection limits for this method were 0.001 $\mu\text{g}/\text{yarn}$ and 0.022 $\mu\text{g}/\text{m}^2$. Recoveries during the field trials ranged from 52 to 104% for filter papers, and from 14 to 26% for yarn samplers. Residue data in this manuscript were not corrected for recovery. Residue data are reported as $\mu\text{g}/\text{yarn}$ for yarn samplers and $\mu\text{g}/\text{m}^2$ for filter paper samplers.

Enzyme Assays

The enzyme assay procedure generally followed previously outlined methods (Ellman et al. 1961; Hooper et al. 1989) but was optimized for butterflies (Bargar 2012a). Cholinesterase activity was measured in homogenates of butterfly head capsules. Head capsules were removed by using a razor blade and weighed to the nearest 0.01 mg. Care was taken to ensure that only the head was placed into the tubes so that only head capsule-associated enzymes were assayed and to minimize additional inhibition from naled contamination on the remaining body. Head capsules for larvae at each location were composited to ensure adequate tissue mass for the assay. Head capsules were placed into a volume of ice-cold buffer (Trizma pH=7.4) equivalent to 99× the head mass and homogenized. The razor blade and homogenizer were rinsed with 95% ethanol and deionized water after each sample to minimize cross contamination. The homogenate was briefly vortexed before removing an aliquot (30 µL) for the assay. Each homogenate was run in triplicate. The butterflies, buffer, and homogenate were kept on ice throughout sample preparation. Cholinesterase activity in head capsules was constant over a range of concentrations (10^{-3} – 10^{-9} M) of the butyryl cholinesterase inhibitor tetraiso-propyl pyrophosphoramidate indicating a lack of butyryl cholinesterase activity. Therefore, future references to ChE activity in this paper are as total ChE activity. The optimal substrate (acetylthiocholine) concentration was 3.16×10^{-3} M while the chromagen (5,5'-dithio-bis-2-nitrobenzoic acid) concentration was 3.23×10^{-3} M. The substrate and chromagen concentrations were in a final volume of 250 µL. Enzyme activity was measured for 2 min (1 reading per 12 s) with absorbance read at 405 nm.

Comparisons

The primary focus of this study was an evaluation of no-spray zone effectiveness in reducing exposure and effects for butterflies in the no-spray zone. Cholinesterase activity (adult and larvae) and mortality (butterfly and mosquitoes) at the locations were averaged among field trials for comparison of the effects in the target area, nontarget area, and reference location. The comparisons were by the parametric one-way ANOVA (post hoc comparisons by the Holm-Sidak) when parametric assumptions were satisfied or by the Kruskal–Wallis one-way ANOVA on ranks when the assumptions were not satisfied. Reference locations were included in the analyses given the possibility that the no-spray zones would not effectively reduce exposure and effects. In addition, ChE for butterflies at the reference location was used to calculate a diagnostic threshold (Hill 1988) to determine whether ChE for individual butterflies in the target and nontarget

areas was unlikely the same as ChE for butterflies at the reference location. Briefly, the diagnostic threshold (DT) is the lower bound of the confidence interval (mean-2SD) for reference organism ChE. If ChE in an organism is less than the DT, then the ChE would be considered depressed. In the present study, the DT was based on the average ChE for butterflies at the reference location. Naled residues on samplers at the locations also were averaged among field trials for comparison between the target and nontarget areas (*t* test, $\alpha=0.05$). Analysis of bivariate plots (nonlinear regression in SigmaPlot 11) between butterfly and mosquito response relative to naled residue on co-located filter paper samplers was used to evaluate the relation between effects and naled exposure. Cholinesterase activity was converted to percent inhibition (relative to ChE of the reference butterflies) for the bivariate plots in facilitate combination of ChE for both species and all field trials in the same plot. Raw data from this study can be accessed through the Department of the Interior Science Base (Bargar et al. 2020).

Results

Detectable naled residues were found on both sampler types in the nontarget area, including on application days when the nontarget area locations were upwind of the modeled deposition areas (Field Trials 1 and 3; Table 2; Fig. 3). In fact, the highest residue levels on filter papers in the nontarget area were detected on a day when winds blew away from the nontarget area (nontarget location 3 during Field Trial 1). Naled residue levels on filter paper samplers in the nontarget area averaged $736 \mu\text{g}/\text{m}^2$ (standard error [SE]=451.0) among the three field trials, whereas those deployed in the target area averaged $2514 \mu\text{g}/\text{m}^2$ (SE=318.7; Fig. 4). The residues on the paper samplers differed significantly between the two areas (1-way ANOVA, $p=0.032$, 4 degrees of freedom [*df*]). Residues on yarn samplers in the target area averaged $19.0 \mu\text{g}/\text{yarn}$ (SE=7.02) and in the nontarget averaged $14.3 \mu\text{g}/\text{yarn}$ (SE=7.42). In contrast to the conclusion for the paper samplers, residue levels on the yarn samplers were not significantly different between the two areas (1-way ANOVA, $p=0.67$, 4 *df*). Importantly, the power level for the two comparisons was less than the convention of 0.8, particularly for the comparison of residues on the yarn samplers (power=0.05), indicating an increased likelihood of incorrectly accepting the null hypothesis that residue levels in the two areas are not different. Regardless of the significance for the comparisons, naled residues were found in the nontarget area.

Butterfly mortality was observed following each aerial application. High adult butterfly mortality at the reference location (60–100% for great southern white, and 11–80%

Table 2 Naled residues on samplers deployed in the nontarget area for each field trial

Field trial	Nontarget location	Distance to nearest swath (meters)	Distance to nearest upwind swath (meters) ^a	Wind direction (degrees)	Naled on filter paper ($\mu\text{g}/\text{m}^2$) ^b	Naled on yarn ($\mu\text{g}/\text{yarn}$) ^b
1	1	139	None	270	1762	33.1
	2	716	None	270	31	0.6
	3	393	None	270	3307	27.4
	4	852	None	270	1146	42.5
2	1	105	105	42	94	BLQ ^c
	2	217	217	42	191	BLQ
	3	134	134	42	247	3.8
	4	359	359	42	2018	59.7
3	1	389	None	300	11	1.2
	2	407	None	300	8	0.2
	3	455	None	300	18	0.4
	4	588	None	300	BLQ ^d	0.1

^aSwath that is directly upwind

^bAverage naled residue on samplers at the respective location

^cBLQ Below the limits of quantitation on yarn, which were 0.6, 3, and 0.001 μg during Field Trials 1, 2, and 3, respectively

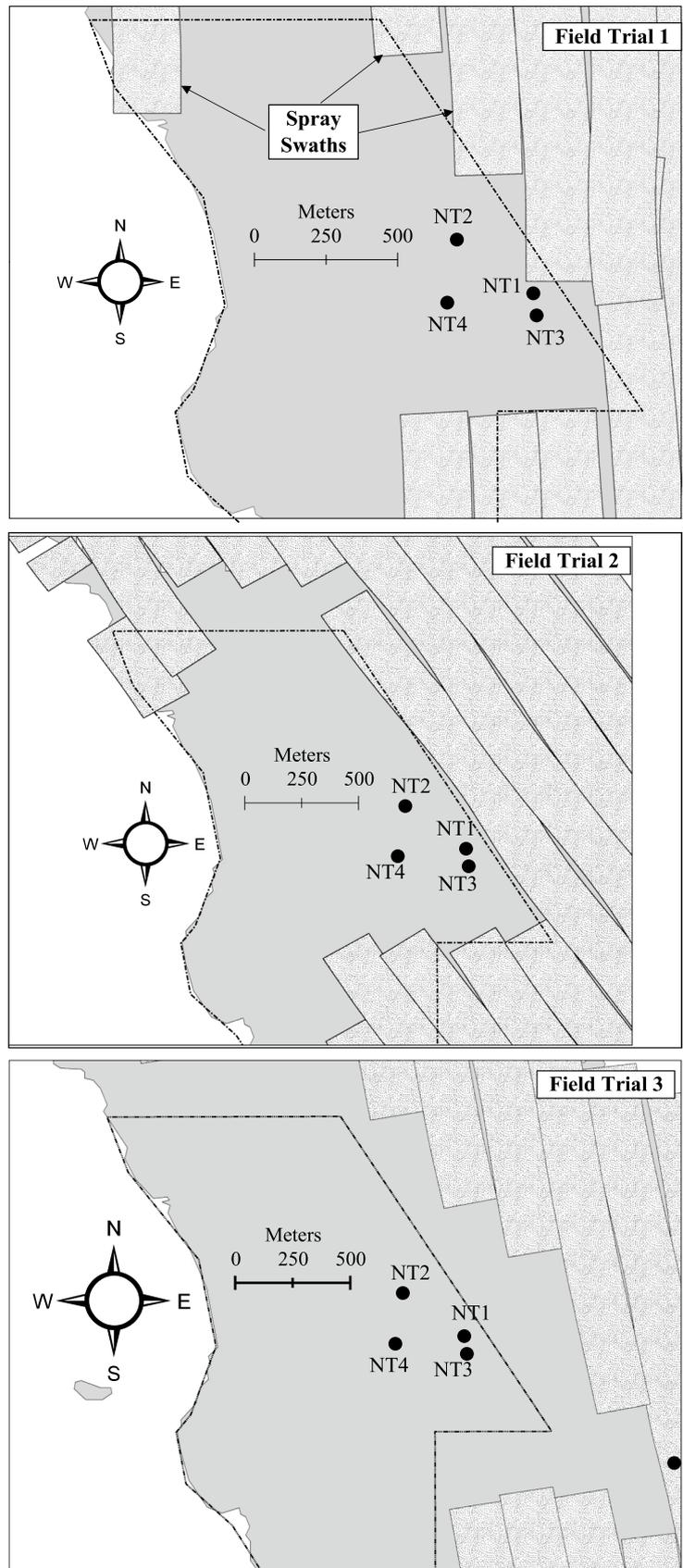
^dBLQ Below the limits of quantitation on filter paper, which were 13.2, 66.3, and 0.022 $\mu\text{g}/\text{m}^2$ during Field Trials 1, 2, and 3, respectively

for the gulf fritillary) indicates that adult butterfly survival was affected at least in part by a factor other than the aerial applications. Starvation was the factor most likely responsible for the elevated reference location mortality. Adult butterfly mortality at the reference location ranged from 60 to 100% during Field Trials 1 and 2 but was 11% during Field Trial 3 when the adults were fed a sugar solution. While fruit was provided during Field Trial 2, the butterflies were not observed to feed on it. Adult butterfly response following the applications will not be discussed further due to the elevated reference area mortality. Larval mortality in the target area ranged from 32–65% and 45–49% for gulf fritillary and great southern white larvae, respectively (Fig. 5). In the nontarget area, mortality ranged from 11–29% and 6–33% for gulf fritillary and great southern white larvae, respectively. The difference between the target and nontarget areas was not significant for the great southern white (Kruskal–Wallis, $p=0.07$, 2 *df*), but it was for the gulf fritillary (1-way ANOVA, $p=0.006$, 2 *df*). With respect to the gulf fritillary larvae, mortality at the reference location was significantly lower relative to that in the target area (Holm–Sidak post hoc, $p=0.002$) but not relative to that in the nontarget area (Holm–Sidak post hoc, $p=0.103$). Despite the difference in average mortality for mosquitoes in the target (82%), nontarget (53%), and reference (23%) areas, the differences were not significant (1-way ANOVA, $p=0.14$, 2 *df*). The power for this comparison (0.24) indicates low ability to detect differences if they existed.

Cholinesterase activity for butterflies typically was highest at the reference location, lowest in the target area and intermediate in the nontarget area (Fig. 6). The differences among the three areas was significant only for larval gulf fritillary butterflies (1-way ANOVA, $p=0.002$, 2 *df*). For the gulf fritillary, ChE at the reference location was significantly greater than ChE in the target area (Holm–Sidak post hoc, $p<0.001$) but not greater than in the nontarget area (Holm–Sidak post hoc, $p=0.142$).

Given the general lack of differences between the target and nontarget areas as well as the detection of naled in the nontarget area, the relation between naled and the measured responses was evaluated by nonlinear regression of naled residues and effects for co-located butterflies and mosquitoes as well as by comparison of ChE in the target and nontarget areas to the DT. Cholinesterase inhibition in butterflies and mortality for mosquitoes and butterfly larvae were both positively related to naled residues on co-located filter paper samplers (Fig. 7). Qualitatively, mosquito mortality was unrelated to naled residues on filter papers up approximately 300 $\mu\text{g}/\text{m}^2$ but then began to increase to near 100% at 500 $\mu\text{g}/\text{m}^2$. A similar change in ChE inhibition and butterfly larval mortality occurred over the same naled residue levels. Coefficients of determination (r^2) for those regressions were 0.41 for larval ChE inhibition and 0.94 and 0.84 for mosquito and larval butterfly mortality, respectively. The average ChE at the reference location among all three field trials was 0.395 (standard deviation [SD]=0.061) and 0.709 $\mu\text{M}/\text{min}^*\text{g}$ (SD=0.061) for gulf fritillary and great southern

Fig. 3 Deployment locations for residue samplers and caged butterflies in the nontarget (NT) area of the National Key Deer Refuge in relation to naled deposition swaths for each of three field trials. Wind directions were 270°, 42°, and 300° for Field Trials 1, 2, and 3, respectively. NT1–4, nontarget area locations 1–4



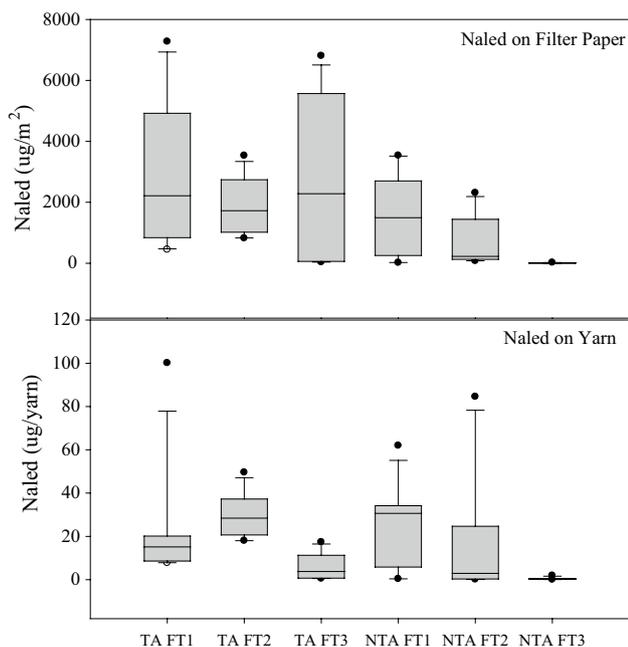
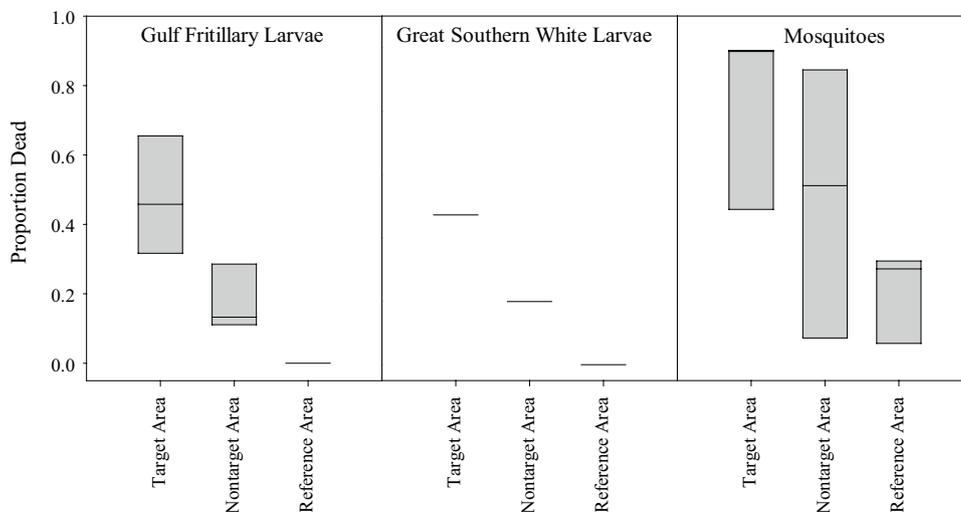


Fig. 4 Box and whisker plots for naled residues on filter paper and yarn samplers during each field trial. Each box represents data for the 4 locations ($n=4$) in either the target area (TA) or nontarget area (NTA) during Field Trials 1 (FT1), 2 (FT2), and 3 (FT3). The top and bottom of each box represent the 75th and 25th percentiles, respectively, whereas the horizontal lines within each box represent the median and the whiskers reflect the 10th and 90th percentiles. The average naled residues on filter papers among the three field trials were significantly higher in the target area relative to the nontarget area (1-way ANOVA, $p=0.032$, 4 df), whereas residues on the yarn samplers were not significantly different between the target and nontarget areas

white larvae, respectively. The respective DTs for those two species were 0.24 and 0.53 $\mu\text{M}/\text{min} \cdot \text{g}$. Cholinesterase activity for larval butterflies at all target area locations was less than the DT, whereas ChE at 25–75% of the nontarget area

Fig. 5 Mortality among three field trials (2 for great southern white) for butterfly larvae and mosquitoes on the National Key Deer Refuge. The top and bottom of each box represent the 75th and 25th percentiles, respectively, while the horizontal lines within each box represent the median. Mortality in the nontarget area was significantly lower relative to the target area only for gulf fritillary larvae (1-way ANOVA, $p=0.006$, 2 df)



locations was less than the DT (Table 3), indicating naled exposure in both areas was sufficient to depress ChE in larval butterflies.

Discussion

The detection of naled residues on samplers deployed in the nontarget area indicated drift of naled residues from the target area. Other studies have reported drift of naled into nontarget areas following aerial naled applications. Zhong et al. (2010), who conducted a study like the present one, reported naled residues on samplers deployed in nontarget areas, including one that was 19 km from the target area. The residue levels at that distant location were adequate to kill all caged mosquitoes but were not adequate to kill co-located butterfly larvae. While they did not explicitly compare residues between the target and nontarget areas, they did report that residue levels on the samplers were significantly related to sampler deployment location. An older study (Hennessey and Habek, 1991, research report to USFWS) with dissimilar application procedures (thermal fogging and a higher application rate) also reported naled residues in no-spray zones. Clearly, drift into no-spray zones have occurred following aerial ULV applications. No-spray zones are established to reduce the risk for organisms in those areas. At the Refuge, they were designated to reduce potential exposure for federally listed species. The present study yielded apparently conflicting indications of the efficacy of the designated areas at reducing exposure. Residues on filter paper samplers were significantly lower in the nontarget area, but residues on the yarn samplers in the nontarget area were not significantly lower, albeit the power for the comparisons were low. Acknowledging that drift into the

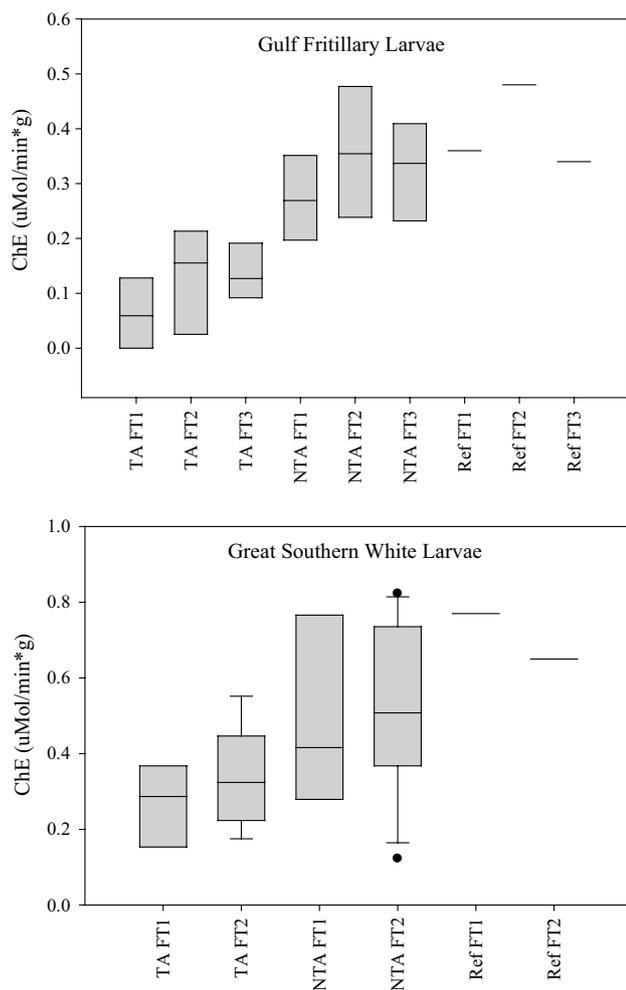


Fig. 6 Box and whisker plots of cholinesterase activity (ChE) for butterfly larvae following aerial naled applications over the National Key Deer Refuge. The top and bottom of each box represent the 75th and 25th percentiles, respectively. The horizontal lines within each box represent the median, while the whiskers represent the 10th and 90th percentiles. Cholinesterase activity averaged among the field trials was significantly higher in the nontarget area relative to the target area only for gulf fritillary butterflies (1-way ANOVA, $p=0.002$, 2 *df*). TA, target area; NTA, nontarget area; FT1, Field Trial 1; FT2, Field Trial 2, FT3, Field Trial 3; Ref, reference location

no-spray zone will occur, the issue is whether that drift is enough to result in significant risk for nontarget organisms.

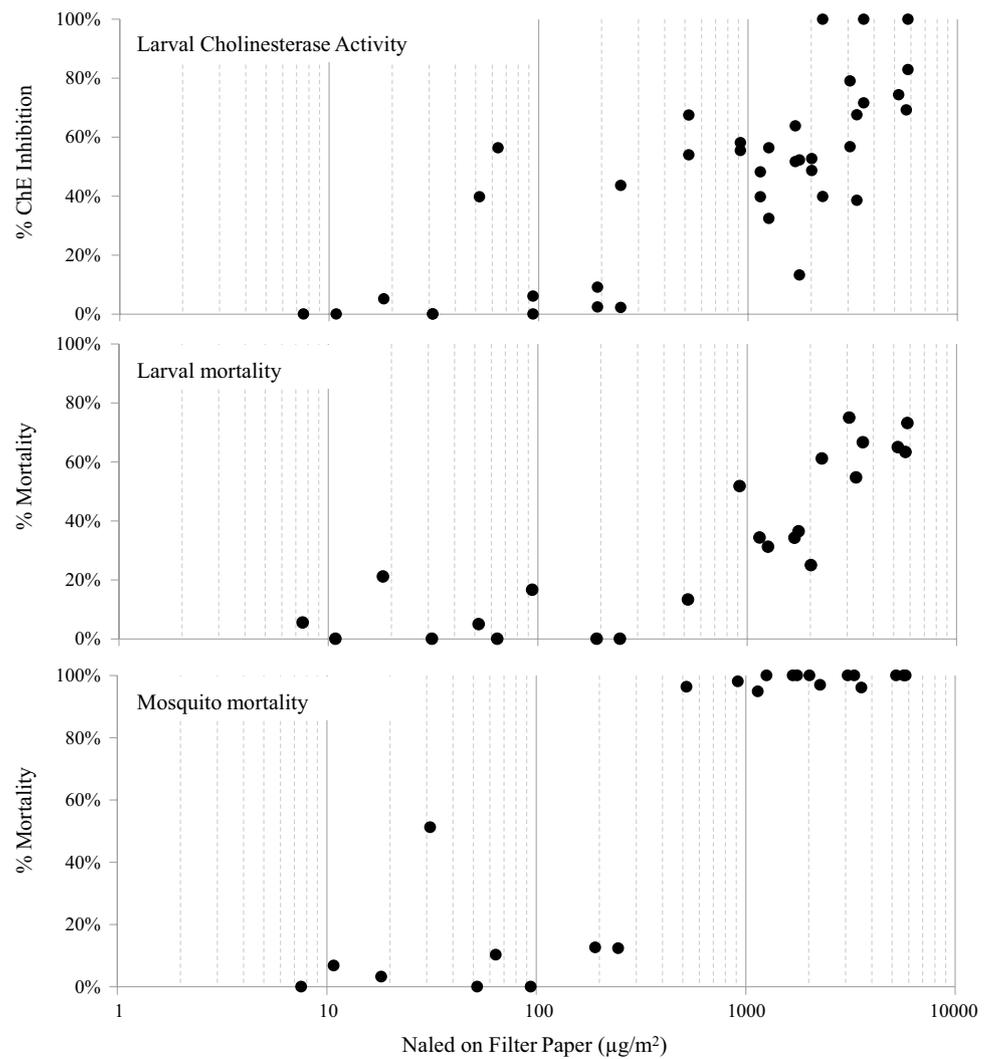
Because naled is categorized as highly toxic to terrestrial invertebrates (USEPA 2006), its drift into the nontarget areas presents a potential risk for the imperiled butterfly species. A few papers have estimated naled risk for butterflies based on residue levels on filter papers. Zhong et al. (2010) related larval Miami blue butterfly mortality to naled residues on filter papers and estimated mortality would be 10% at residue levels of $1000 \mu\text{g}/\text{m}^2$. Bargar (2012b) equated naled residues on filter papers to mortality of adult butterflies. Deposition levels of $20\text{--}120 \mu\text{g}/$

m^2 approximated the estimated 10th percentile LD50 for adult butterflies, depending on the family, whereas levels of $210\text{--}1040 \mu\text{g}/\text{m}^2$ approximated the 90th percentile LD50. Hoang and Rand (2015) presented LD50s for adult butterflies as mass of chemical per unit surface area, which can be related to naled deposition onto filter papers. They estimated the 10th percentile LD50 to be $123 \mu\text{g}/\text{m}^2$, which is in line with the 10th percentiles estimated by Bargar (2012b). The average residue level on filter papers in the target area ranged from 1882 to $2898 \mu\text{g}/\text{m}^2$ and in the nontarget area ranged from 9 to $1562 \mu\text{g}/\text{m}^2$. Based on the published risk estimates and the residue levels measured during the present study, effects on adult and larval butterflies are expected in the target area. While measured residue levels in the nontarget area are lower relative to the target area, they were high enough during Field Trials 1 and 2 to expect effects for at least adult butterflies. However, the likelihood of effects in the nontarget area following any one application is variable given the variability among applications of residues in the nontarget area measured during the present study.

The results from the field trials indicated enough risk to butterflies to support the Refuge's decision to expand nontarget areas for protection of habitat critical for their conservation (USFWS 2014). These trials provided information that could be used for adaptive management by both the mosquito control district and the Refuge system, which both have an interest in evaluating the accuracy of treatments. In addition, they provided information that could be used to strike a balance between the need for protection against vector-borne diseases in human populations and the conservation needs of imperiled lepidoptera. However, these trials do highlight the need for continued testing of pesticide application accuracy.

The present study measured ChE activity and mortality in surrogate butterflies deployed in the target and nontarget areas during three typical aerial applications to determine the effectiveness of the no-spray zones at protecting imperiled butterflies. Larval butterfly mortality was high in the target and nontarget areas following the aerial applications, but the difference between the areas was significant only for gulf fritillary butterflies. Cholinesterase activity for butterflies in the nontarget area was depressed following the applications, and as was the case for mortality, the difference between the areas was significant only for the gulf fritillary butterflies. Therefore, the measured effect data did not yield consistent information about the no-spray zone effectiveness at protecting butterflies. This is likely due to the amount of spray drift into the no-spray zone and the resulting effects on the butterflies. Zhong et al. (2010), on the other hand, reported that no-spray zones effectively reduced effects in butterflies. Their study reported that Miami blue larvae mortality was lower in the nontarget area (referenced to as "drift zone" in

Fig. 7 Bivariate plots of percent cholinesterase (ChE) inhibition or percent mortality relative to naled residues on co-located filter paper samplers for all three field trials. Each dot represents the average effect at a location relative to naled residues on the co-located filter paper sampler



their paper). Except for one location that was 19 km from the target area, the distances of the other two nontarget area locations from the target area were not reported. However, based on Fig. 1 of their study, those other two locations appear to have been further from the target area than were

Table 3 Proportion of larval butterflies with cholinesterase activity less than the diagnostic threshold^a

	Gulf Fritillary		Great Southern White	
	TA	NTA	TA	NTA
Field Trial 1	1.0	0.5	1.0	0.75
Field Trial 2	1.0	0.25	1.0	0.25
Field Trial 3	1.0	0.25		

^aDiagnostic threshold = mean reference cholinesterase activity - 2*SD for reference cholinesterase activity

TA target area, NTA nontarget area

the nontarget locations from the target area of the present study. Given that droplet deposition declines with distance from the targeted spray area (Teske et al. 2000), the greater distances from the target area in their study should result in the lower exposures and reduced effect levels they reported for the nontarget area. Comparisons of studies evaluating no-spray zone effectiveness at reducing risk need to consider the distances of the nontarget area test locations relative to the areas targeted by the applications. Organisms at nontarget area test locations relatively close to the target area are more likely to be affected because of drift, whereas those at test locations relatively far from the target area are less likely to be affected, because exposure to drift will be minimized. A conclusion regarding the nontarget area effectiveness will differ because of the choice of nontarget area test locations. Studying a no-spray zone's effectiveness at reducing risk should evaluate response of organisms throughout the entire no-spray zone because focusing on one area biases the results and their interpretation.

Data from the present study indicate that naled aerially applied by ULV sprays will drift from target areas on the Refuge into the no-spray zones. The small drop-let sizes typical of ULV sprays enhance efficacy of the sprays against mosquitoes (Sugiura et al. 2011; Harburger et al. 2012) but also enhance drift (Teske et al. 2000; Lothrop et al. 2007; reviewed in Hilz and Vermeer 2013). Drift was a concern for resource managers responsible for conservation of sensitive species in the no-spray zones, because it could adversely affect those species. Indeed, adverse effects were measured during the present study for surrogate butterflies in the nontarget or no-spray zone ultimately leading to the expansion of no-spray zones on the Refuge (USFWS 2014). While the data were variable among the field trials, they do indicate the inconsistent effectiveness of the no-spray zones at reducing risk for the imperiled butterflies. Further research is necessary to assist natural resource managers balancing the needs for mosquito control with conservation of imperiled species susceptible to mosquito control insecticides.

Conclusions

Imperiled species conservation is a responsibility for the National Key Deer Wildlife Refuge. Given its location in a subtropical climate and the widespread salt marsh habitat in the region, the Refuge often harbors dense mosquito populations that may present a human health risk and necessitates mosquito control. Conservation of imperiled species susceptible to mosquito control insecticides is problematic in an area subject to mosquito control practices. The present study was conducted to determine the effectiveness of one conservation practice, no-spray zones, at reducing the risk from vector control. Permitted aerial applications over the Refuge resulted in detectable naled residues on the Refuge including in the no-spray zones, and those residue levels exceeded published toxicity data for butterflies indicating the applications could lead to significant risk for butterflies. That risk was further indicated by elevated mortality and ChE inhibition for butterfly larvae deployed in the no-spray zones during the applications. While the effects measured for butterflies reflected the ineffectiveness of the no-spray zone at protecting the butterflies, variability among the applications indicated the need for further research into resource conservation measures in areas subject to adult mosquito control practices.

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