

Hazard/Risk Assessment

USE OF BUTTERFLIES AS NONTARGET INSECT TEST SPECIES AND THE ACUTE TOXICITY AND HAZARD OF MOSQUITO CONTROL INSECTICIDES

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Abstract—Honeybees are the standard insect test species used for toxicity testing of pesticides on nontarget insects for the U.S. Environmental Protection Agency (U.S. EPA) under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA). Butterflies are another important insect order and a valued ecological resource in pollination. The current study conducted acute toxicity tests with naled, permethrin, and dichlorvos on fifth larval instar (caterpillars) and adults of different native Florida, USA, butterfly species to determine median lethal doses (24-h LD50), because limited acute toxicity data are available with this major insect group. Thorax- and wing-only applications of each insecticide were conducted. Based on LD50s, thorax and wing application exposures were acutely toxic to both caterpillars and adults. Permethrin was the most acutely toxic insecticide after thorax exposure to fifth instars and adult butterflies. However, no generalization on acute toxicity (sensitivity) of the insecticides could be concluded based on exposures to fifth instars versus adult butterflies or on thorax versus wing exposures of adult butterflies. A comparison of LD50s of the butterflies from this study (caterpillars and adults) with honeybee LD50s for the adult mosquito insecticides on a $\mu\text{g}/\text{organism}$ or $\mu\text{g}/\text{g}$ basis indicates that several butterfly species are more sensitive to these insecticides than are honeybees. A comparison of species sensitivity distributions for all three insecticides shows that permethrin had the lowest 10th percentile. Using a hazard quotient approach indicates that both permethrin and naled applications in the field may present potential acute hazards to butterflies, whereas no acute hazard of dichlorvos is apparent in butterflies. Butterflies should be considered as potential test organisms when nontarget insect testing of pesticides is suggested under FIFRA. Environ. Toxicol. Chem. 2011;30:997–1005. © 2011 SETAC

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INTRODUCTION

Six insecticides (pyrethroids: permethrin, resmethrin, sumithrin, and organophosphates: naled, malathion, fenthion) are used as adult mosquito control agents in the United States [1]. In Monroe County (Florida Keys, FL, USA), the Mosquito Control District predominately uses naled and permethrin as an ultra-low-volume spray for year-round adulticiding treatments by aerial (e.g., naled) or ground (truck-mounted) application (e.g., permethrin) based on recent mosquito control usage data, by acreage applied, to control one or more of the 74 nuisance and disease vector mosquito species [2].

Naled is an organophosphate insecticide that acts as a nerve poison by blocking synaptic transmission in the cholinergic parts of the nervous system by binding to the active site of acetyl cholinesterase, where it inhibits the enzyme from breaking down the neurotransmitter acetylcholine [3]. Naled is rapidly broken down (<3 d) on plant surfaces, in air, water, and soil/sediment [4]. Its main degradation product, dichlorvos, is also an organophosphate insecticide that rapidly degrades, is of biological importance, and is typically considered in risk assessments for naled [5]. Permethrin, like all synthetic pyrethroids, is also neurotoxic, because it changes sodium ion permeability in nerve cells during the excitatory phase of the action potential, thus affecting repetitive activity in the sensory and motor pathways [6]. Although naled is rapidly broken down in the

environment by hydrolysis, permethrin can persist in sediment, soil, and plant tissue for weeks to months [7–11].

Under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA), the U.S. EPA classifies naled, dichlorvos, and permethrin as highly toxic insecticides to aquatic organisms (e.g., fish, nonmollusk invertebrates) and honeybees based on acute toxicity data. The honeybee (*Apis mellifera* L.) is the standard nontarget insect species required for testing by chemical registrants by the U.S. EPA under FIFRA. Butterflies are not used as nontarget insect test species under FIFRA.

Although not used as a nontarget insect test group, the fact that insecticides are toxic to adult butterflies and caterpillars has long been recognized ([12–17]; <http://www.epa.gov/essp/litstatus/effects/redleg-frog/permethrin/appendix-k.pdf>). Exposure to nontarget organisms can and will occur from drift from aerial and ground applications of pesticides miles away from target sites. However, exposure to nontarget species from aerial application is far more significant than application by ground equipment, which results in more limited exposure [18]. Even with tractor-mounted equipment operating under normal application conditions, spray drift was estimated at approximately 5 to 10% of the active ingredient for a single swath [18–20].

In terms of species numbers, Hymenoptera (ants, bees, wasps, and sawflies) and Lepidoptera (butterflies and moths) are among the four largest insect orders, along with Coleoptera (beetles) and Diptera (flies). All four orders play a role in pollination, although adult lepidoptera are mainly significant in pollination of flowers and of little commercial importance in the pollination of crops [21]. The main ecological importance of Lepidoptera is that their larvae stages (caterpillars) and to some extent the adults eat and breakdown plant tissue (they play a role

All Supplemental Data may be found in the online version of this article.

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in nutrient cycling) and that all stages of this order are prey for insectivorous predators, such as birds, bats and other small mammals, and parasitoids). As a predominately phytophagous group, they are important components of terrestrial systems and are economically important as pests in agriculture and forestry. Over 150,000 described species of Lepidoptera exist, with 18,000 butterfly species [22]. Lepidoptera have been used as model systems in developmental and molecular biology, physiology, evolution, and ecology [23].

Butterfly populations may be potentially exposed during their larval stages and as adults to both single and multiple insecticide applications, resulting in ecological risks from direct spraying or from indirect residual deposits on plant tissue [24]. For example, butterflies inhabiting farmland become exposed to insecticides when their field margin habitats are contaminated by direct overspray, spray drift, and from volatilization [25]. Larval periods for common butterflies can coincide with timing of insecticide use to control pests [12]. Although not addressed in this study, herbicides also may indirectly affect butterfly populations through direct mortality/elimination of their larval food plants or by reducing the number of nectar-bearing flowers for ingestion by adults [26,27].

The acute toxicity database with butterflies is limited for naled and permethrin exposures [28]. In addition, no toxicity data are available for dichlorvos exposures and butterflies. This gains in importance because, after application and drift, adult mosquito insecticide exposures (quantity of chemical in contact with total body surface area) to butterflies are likely to be significantly higher than those to honeybees, because of their large surface areas. Longley and Sotherton [29] point out that “a greater range of butterfly species need to be screened to provide a useful ranking of intrinsic susceptibilities of butterfly species against a range of pesticides.”

This study is organized into four themes: description of methodology used to evaluate the acute toxicity of naled, dichlorvos, and permethrin to larval (fifth-instar caterpillars) and adult native Florida butterflies; characterization of the acute toxicity (e.g., 24-h LD50s) of these three insecticides using survival of larvae and adult butterflies after topical exposures to the thorax and wings; characterization of the toxic effects data relative to species sensitivity distributions (SSDs) for each insecticide, and summary of the importance of using butterflies in addition to honeybees in toxicity testing of nontarget insects to pesticides.

MATERIALS AND METHODS

Test organisms

Butterflies are diurnal insects with clubbed antennae, three main body parts (head, thorax, and abdomen) (Fig. 1), and a simple life cycle (egg→larvae (caterpillar)→pupae (chrysalis)→adult). Antennae are used for balance, motion, and chemoreception. The thorax has three segments: the prothorax, mesothorax, and metathorax, with each segment containing one pair of legs. Two sets of wings (two forewings and two hindwings) are attached to the second and third thoracic segments (meso- and metathorax). Tubular veins nourish and support the wings. Besides flight, wings are important in courtship and respiration (oxygen exchange). Butterflies have complete metamorphosis through a life cycle (one generation) that consists of four stages: egg, larva (caterpillar), pupa (chrysalis), and adult. More information on the anatomy, physiology, ecology, molecular biology, and genetics of Lepidoptera can be found in other studies [21–23,30].

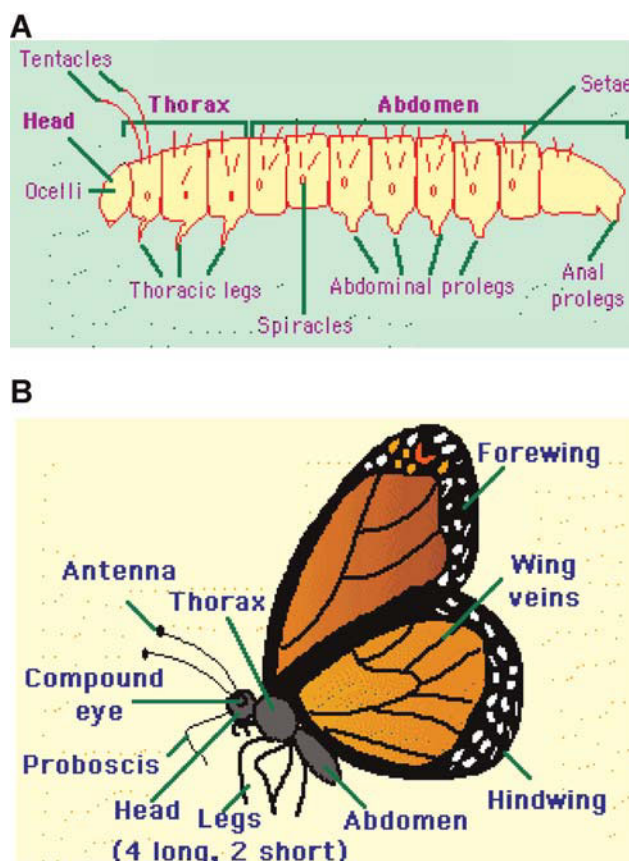


Fig. 1. Anatomy of caterpillar (A) and butterfly (B) (<http://www.enchantedlearning.com/subjects/butterfly>).

Larvae and adult butterflies were the experimental units in the current study, because both stages are potential targets of exposure after direct spraying of mosquito control agents. Caterpillars may rest and feed on exposed host plants, and adults are potentially exposed during flight, resting on exposed areas of plants or ingesting contaminated nectar. Based on the rapid degradation of naled and dichlorvos, acute exposure from direct spray may be the primary route of exposure for larval and adult butterflies, and long-term dermal contact (or ingestion of plant tissue and nectar) from host plants should be minimal. However, for permethrin, based on a longer environmental half-life, acute and chronic exposures may potentially occur to both larvae and adult butterflies.

Five Florida native butterflies were used in the current study: common buckeye (*Junonia coenia*), painted lady (*Vanessa cardui*), zebra longwing (*Heliconius charitonius*), atala hair-streak (*Eumaeus atala*), and white peacock (*Anartia jatrophae*). Two life stages of butterflies (adult and fifth-instar caterpillar) were obtained from a commercial grower. Holding and testing were conducted in the greenhouse of the Non-Target Insect Laboratory of the Ecotoxicology and Risk Assessment Laboratory, Florida International University, North Miami, Florida, USA. Before testing, caterpillars were held in 500-ml plastic cups with host plants. Adult butterflies were held in butterfly cages (60 × 60 × 100 cm) and fed Gatorade® before testing. Butterflies and caterpillars were held at a laboratory temperature of 26 ± 2°C, and a humidity of over 50% for a minimum of 2 to 3 h before test initiation. Caterpillars and butterflies were not fed during the study.

Exposure and toxicity testing

Mosquito control insecticides used in this study were technical grade permethrin, Dibrom (naled), and the metabolite of naled (dichlorvos). Naled and dichlorvos were purchased from Sigma-Aldrich. Permethrin consisted of 40% *cis*-permethrin isomer and 60% *trans*-permethrin isomer and was obtained from FMC Corporation. Exposure solutions were prepared using acetone as a solvent.

Each toxicity test consisted of five treatment concentrations, one untreated control (no exposure), and one solvent control (acetone as solvent exposure). For the adult butterflies of all five species and the fifth-instar caterpillars of common buckeye, atala hairstreak, and white peacock, each treatment concentration contained three replicates with 10 organisms each. The fifth-instar caterpillars of painted lady and zebra longwing are cannibalistic. Therefore, each treatment concentration contained 10 replicates for each treatment concentration with one caterpillar per replicate. Knowledge of the behavioral ecology of each species is critical to conducting butterfly tests. For cannibalistic caterpillars, test chambers were 300-ml plastic cups (Instaware). For non-cannibalistic caterpillars, test chambers were 1-L plastic beakers (Cole Parmer). For adult butterflies, test chambers were 30-cm³ butterfly cages (Live Monarch Foundation Significant Solutions).

Thorax- and wing-only exposure approaches were used in the current study (Fig. 2). Before exposure initiation, 20 organisms (caterpillars and adult butterflies) were randomly selected and weighed on a Mettler Toledo Balance (model AB204-S, Mettler-Toledo) to determine body weight. During weighing, live caterpillars and adult butterflies were held in 400-ml plastic beakers and glassine envelopes, respectively. At test termination, 20 adult butterflies from control treatments were randomly collected in a glassine envelope and killed in a freezer for determining total body surface area, including wings.

Thorax-only exposure was conducted with each species following previously published methods ([28,31–35]; J.E. Eliazar, 1992, MS thesis; M.H. Salvato, 1999, MS thesis, University of Florida, Gainesville, FL, USA). Exposure was conducted by applying a single application of 1 μ l exposure solution onto the dorsum of the thorax. An adjustable micropipette with a volume range of 0.1 to 2 μ l was used for dosing (Fisher Scientific). The solution was allowed to air dry for 15 s, and the organism was then transferred into test chambers. Wing-only exposure was conducted by applying 0.5 μ l exposure solution onto the front of the left and right forewings. Because butterfly wings have a condensed vein net, the applied solution (acetone as solvent) was rapidly absorbed on wing tissue regardless of whether the applied area contained veins. The solution was allowed to air dry for 15 s before transferring organisms into test chambers.

Data on total body weight for caterpillars and adults, and on total body surface area for adults, were used to calculate lethal doses (24-h LD50s) in microgram insecticide per gram body weight (μ g/g) and microgram insecticide per square meter of butterfly total body surface area (μ g/m²), respectively. Total body surface area is defined as the top surface of the head, thorax, abdomen, and wings. One side (top surface) of the total body surface area of the butterfly (head, thorax, abdomen, and wings) was assumed to be the initial part exposed to drift after application and involved in the toxification process. In addition, LD50s in μ g insecticide/m² units are needed to compare with the field exposure data (drift deposition) to conduct a hazard assessment when exposure is considered, because concentra-

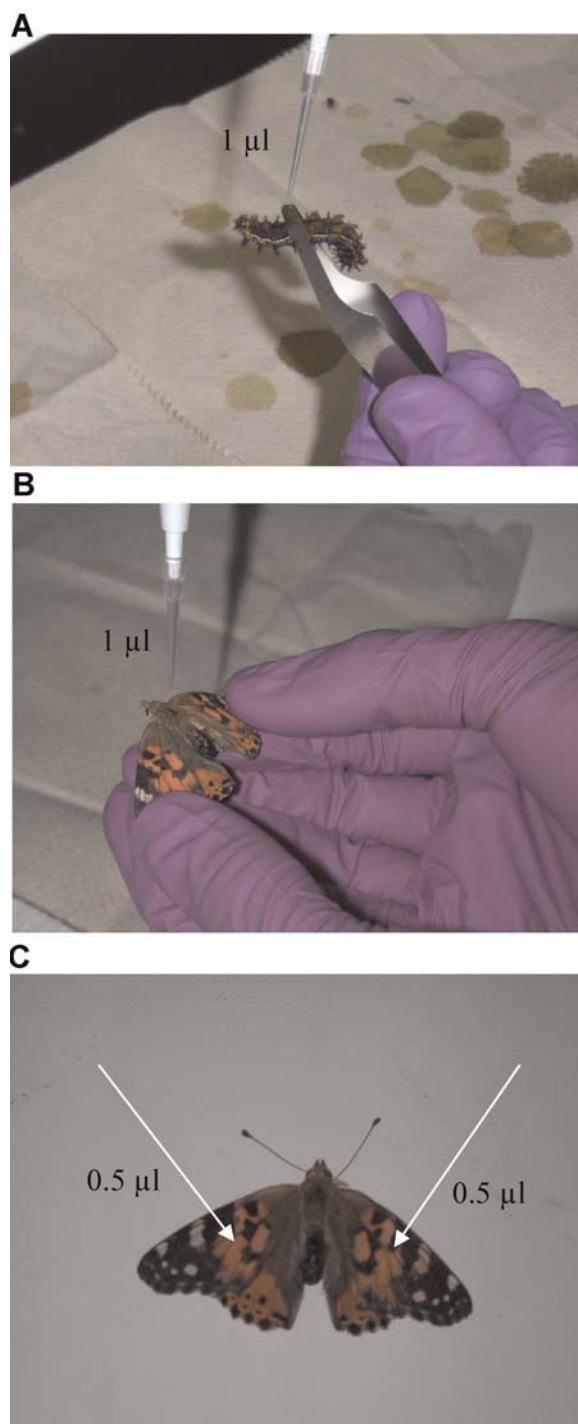


Fig. 2. Exposure technique. (A) Exposure on caterpillar thorax; (B) exposure on butterfly thorax; (C) exposure on butterfly forewings.

tions of the insecticides are typically measured in the field on filter pads or plant foliage in these units. Wing surface area was measured with the body by keeping the natural overlapping of the wing costal margins open as near as possible to their position during flight, then tracing it onto graph paper to determine total body surface area. Total body surface area was measured by counting numbers of squares traced onto graph paper. More details about morphological measurement of butterflies can be found in Dudley [36] and Srygley and Dudley [37].

Survival of caterpillars and adult butterflies was measured at 24 h postexposure for calculating lethal effect doses (24-h

LD50). Spearman-Kärber and probit methods were used to calculate 24-h LD50 values. All toxicity tests were conducted under a photoperiod of 12:12 h light:dark cycle, at $26 \pm 2^\circ\text{C}$ and humidity between 50 and 80%. Concentrations of insecticide exposure solutions were measured at Mote Marine Laboratory (Sarasota, FL, USA) for calculating 24-h LD50 values.

A hazard quotient (HQ) approach (where HQ = highest observed environmental concentration/lowest LD50 concentration or 10th percentile from the SSD) was used to characterize potential hazard of permethrin, naled, and dichlorvos to adult butterflies from adult mosquito control application. The highest measured observed exposure concentrations after application of permethrin and naled were obtained from actual field studies by Pierce [38] for permethrin, naled, and dichlorvos postapplication exposures and of Zhong et al. [39] for naled postapplication exposures only. The HQ is based on a comparison of only a single highest concentration from drift deposition (on a filter pad or foliage) after application, when in fact multiple spray applications on consecutive days or on different days in the same week can and do occur based on mosquito pressure.

RESULTS AND DISCUSSION

Total body weight and surface area of butterfly species

Results of total body weight and total body surface area are shown in Table 1. Body weight ranged from 0.299 g (zebra longwing) to 0.553 g (painted lady) and from 0.096 g (white peacock) to 0.213 g (painted lady) for fifth-instar caterpillars and adult butterflies, respectively. Total body weight of five Florida native adult butterfly species in this study are within the range of total body weight of other butterfly species reported by Srygley and Dudley [37]; 0.036 g (*Itaballia demophoon*) to 0.640 g (*Eurytides ilus*). Body weight of the five Florida native caterpillar species in the current study was two to four times higher than the body weight of caterpillars from Srygley and Dudley [37]. Weight of chrysalis and liquid lost during hatching would account for the difference between adult butterflies and their caterpillar weights.

Among the five Florida native butterfly species in this study, zebra longwing had the largest total body surface area ($1.320 \times 10^{-3} \text{ m}^2$). Area of the atala hairstreak was the smallest ($0.639 \times 10^{-3} \text{ m}^2$). Total wing surface area of other butterfly species reported by Betts and Wootton [40] (e.g., $4.5 \times 10^{-3} \text{ m}^2$ for *Troides rhadamantus*, $2.4 \times 10^{-3} \text{ m}^2$ for *Pachliopta hector*) was higher than the total body area of the butterflies in the current study. However, Vilorio et al. [41] found that wing surface area of *Redonda bordoni*, *R. empetrus empetrus*, *R. empetrus bolivari*, *R. empetrus* ssp., and *Redonda* sp. nov. was less than $0.8 \times 10^{-3} \text{ m}^2$ [41]. Total body surface area of the butterfly species in the current study were within the range of

total body area of other butterfly species reported by Srygley and Dudley, such as $0.186 \times 10^{-3} \text{ m}^2$ (*Itaballia demophoon*) to $3.6 \times 10^{-3} \text{ m}^2$ (*Morpho amathonte*) [37].

Toxicity of insecticides—thorax exposure

Toxicity of the three insecticides to caterpillar and adult butterfly species via thorax exposure is shown in Table 2. Control mortality (include solvent and negative controls) for adult butterflies in all tests was less than 10%, except for one test with zebra longwing (14%), whereas control mortality in caterpillars was less than 3% in all tests. For all five larval species and thorax exposure, the 24-h LD50 values for permethrin, naled, and dichlorvos ranged from 0.08 to $0.79 \mu\text{g/g}$, 0.19 to $10.82 \mu\text{g/g}$, and 1.13 to $7.36 \mu\text{g/g}$, respectively. For all five adult species and thorax exposure, the 24-h LD50 values for permethrin, naled, and dichlorvos ranged from 0.18 to $1.6 \mu\text{g/g}$, 0.90 to $30.08 \mu\text{g/g}$, and 1.48 to $11.30 \mu\text{g/g}$, respectively.

In general, permethrin was more toxic than naled and dichlorvos to caterpillars and adult butterflies. The 24-h LD50 values for permethrin were approximately 27 and 32 times lower than the 24-h LD50 values for naled and dichlorvos, respectively.

Naled, fenthion, malathion, and resmethrin (Scourge) were tested on nontarget lepidopteran larvae of giant swallowtail, *Papilio cressphontes* (Papilionidae; relative of the Schaus' swallowtail), painted lady, *Vanessa cardui* (Nymphalidae), zebra longwing, *Heliconius charitonius* (Nymphalidae), Gulf fritillary, *Agraulis vanillae* (Nymphalidae), and on the adults of *P. cressphontes* and *V. cardui* [42]. All these species occur in south Florida and the Florida Keys. The LD50s for all species occurred at concentrations less than $1.0 \mu\text{g}$ active ingredient (a.i.) per gram body weight. Based on LD50s, acute toxicity decreased in the following order: resmethrin > naled > fenthion > malathion, but no generalization could be concluded about the sensitivity of different life stages. Results for LD50 toxicity studies by Eliazar and Emmel for naled and fifth instars of *Papilio cressphontes* and *Agraulis vanilla* were similar to our LD50 results for zebra longwing and white peacock [42]. However, except for the zebra longwing, the adult butterfly LD50s in the present study for naled were significantly higher than those of Eliazar and Emmel [42].

Salvato also conducted toxicity tests with Florida native butterfly species at different life stages and malathion, naled, and permethrin [28]. Based on LD50s, acute toxicity decreased in the following order: permethrin > naled > malathion, but no generalization could be concluded about the sensitivity of different life stages. Similar to the study by Eliazar and Emmel, pyrethroids were the most toxic adult mosquito insecticides to butterflies [42].

Table 1. Total body weight and body surface area of five Florida, USA native butterfly species (mean $\pm$ standard deviation, $n = 20$)

Life stage	Species	Total body wt (g)	Total body surface area (10^{-3} m^2)
Fifth-instar caterpillars	Atala hairstreak	0.362 ± 0.013	— ^a
	Zebra longwing	0.299 ± 0.068	— ^a
	Common buckeye	0.423 ± 0.029	— ^a
	Painted lady	0.553 ± 0.050	— ^a
	White peacock	0.425 ± 0.012	— ^a
Adult butterfly	Atala hairstreak	0.117 ± 0.017	0.639 ± 0.009
	Zebra longwing	0.133 ± 0.017	1.320 ± 0.006
	Common buckeye	0.106 ± 0.018	0.854 ± 0.076
	Painted lady	0.213 ± 0.003	0.960 ± 0.013
	White peacock	0.096 ± 0.010	0.986 ± 0.008

^a Not measured.

Table 2. Acute toxicity of permethrin, naled, and dichlorvos to Florida native butterflies as a result of exposure on the thorax^{a,b}

Compounds	Life stage	Species	Exposure on thorax		
			24-h LD50 ($\mu\text{g/g}$)	24-h LD50 ($\mu\text{g/m}^2$)	24-h LD50 ($\mu\text{g/organism}$)
Permethrin	Fifth-instar caterpillars	Atala hairstreak	0.08 (0.07–0.09)	— ^c	0.03 (0.02–0.04)
		Zebra longwing	0.11 (0.05–0.18)	— ^c	0.03 (0.02–0.05)
		Common buckeye	0.23 (0.18–0.29)	— ^c	0.09 (0.07–0.12)
		Painted lady	0.46 (0.29–0.74)	— ^c	0.26 (0.16–0.41)
	Adult butterflies	White peacock	0.79 (0.62–1.00)	— ^c	0.34 (0.27–0.43)
		Atala hairstreak	1.60 (1.05–2.42)	307 (202–467)	0.20 (0.13–0.30)
		Zebra longwing	0.18 (0.14–0.24)	21 (16–27)	0.03 (0.02–0.04)
		Common buckeye	1.07 (0.80–1.42)	145 (109–193)	0.12 (0.09–0.17)
	Fifth-instar caterpillars	Painted lady	1.10 (0.97–1.26)	247 (216–281)	0.24 (0.21–0.27)
		White peacock	0.74 (0.54–1.03)	64 (46–88)	0.06 (0.05–0.09)
		Atala hairstreak	— ^d	— ^d	— ^d
		Zebra longwing	0.45 (0.07–1.32)	— ^c	0.14 (0.02–0.40)
Naled	Adult butterflies	Common buckeye	4.04 (3.31–4.94)	— ^c	1.84 (1.51–2.25)
		Painted lady	10.82 (8.20–14.27)	— ^c	5.98 (4.53–7.89)
		White peacock	0.19 (0.14–0.26)	— ^c	0.49 (0.41–0.58)
		Atala hairstreak	28.22 (21.70–36.69)	4,285 (3,295–5,572)	2.74 (2.11–3.56)
	Fifth-instar caterpillars	Zebra longwing	0.90 (0.40–1.37)	84 (38–127)	0.11 (0.05–0.17)
		Common buckeye	6.84 (5.45–8.57)	929 (741–1164)	0.79 (0.63–1.00)
		Painted lady	30.08 (27.88–32.45)	6,579 (6,099–7,098)	6.32 (5.86–6.81)
		White peacock	14.68 (9.01–23.99)	1,522 (933–2,483)	1.50 (0.92–2.45)
	Adult butterflies	Atala hairstreak	1.63 (1.30–2.05)	— ^c	0.58 (0.46–0.72)
		Zebra longwing	1.57 (1.23–2.00)	— ^c	0.47 (0.37–0.60)
		Common buckeye	7.36 (6.10–8.90)	— ^c	2.99 (2.48–3.61)
		Painted lady	3.79 (2.62–5.46)	— ^c	2.09 (1.45–3.02)
Dichlorvos	Adult butterflies	White peacock	1.13 (0.94–1.35)	— ^c	0.49 (0.41–0.58)
		Atala hairstreak	6.56 (5.74–7.50)	1,336 (1,169–1,526)	0.85 (0.75–0.96)
		Zebra longwing	1.56 (0.30–2.49)	145 (28–232)	0.19 (0.04–0.31)
		Common buckeye	11.30 (6.02–21.20)	1,125 (599–2,111)	0.96 (0.51–1.80)
	Adult butterflies	Painted lady	4.66 (4.00–5.43)	1,044 (896–1,217)	1.00 (0.86–1.17)
		White peacock	1.48 (1.19–1.86)	153 (123–192)	0.15 (0.12–0.19)
		Atala hairstreak	—	—	—
		Zebra longwing	—	—	—
		Common buckeye	—	—	—
		Painted lady	—	—	—
		White peacock	—	—	—

^a LD50 = median lethal dose.^b Data in parentheses are 95% confidence interval.^c Not measured.^d Not studied.

The LD50 values found in the present study for thorax exposure (for atala hairstreak and naled and permethrin, and for the zebra longwing and permethrin) were significantly higher than the LD50 values reported by Salvato [28]. However, butterfly test species used by Salvato [28] were collected from the field, where they may have been previously exposed to insecticides because of mosquito control operations in the area where they were found. In addition, in tests by both Salvato [28] and Eliazar and Emmel [42], the controls consisted of exposure to No. 2 diesel fuel [28] or a mixture of No. 2 diesel fuel plus fog oil [42], whereas in the present study we had both untreated controls and acetone controls.

Sensitivity between species

For permethrin, atala hairstreak and zebra longwing fifth-instar caterpillars were more sensitive than common buckeye, painted lady, and white peacock fifth-instar caterpillars. The 24-h LD50 values were approximately three to nine times lower for atala hairstreak and zebra longwing fifth-instar caterpillars than for buckeye, painted lady, and white peacock fifth-instar caterpillars (Table 2). However, with adult butterflies, zebra longwing and white peacock were more sensitive than atala hairstreak, common buckeye, and painted lady. The 24-h LD50 values were up to eight times higher for atala hairstreak, common buckeye, and painted lady than for zebra longwing and white peacock.

For naled, among the four species in this study, fifth-instar caterpillar white peacock was the most sensitive species, with a

24-h LD50 of 0.19 $\mu\text{g/g}$, but zebra longwing was the most sensitive adult butterfly, with a 24-h LD50 of 0.90 $\mu\text{g/g}$. Painted lady was the most tolerant species, with a 24-h LD50 of 10.82 $\mu\text{g/g}$ for fifth-instar caterpillars and 30.08 $\mu\text{g/g}$ for adult butterflies. For dichlorvos, white peacock was the most sensitive species regardless of life stage. The 24-h LD50 values for white peacock were 1.13 $\mu\text{g/g}$ and 1.48 $\mu\text{g/g}$ for the fifth-instar caterpillar and adult butterfly, respectively. Common buckeye was the most tolerant species. The 24-h LD50 values for common buckeye were 7.36 $\mu\text{g/g}$ and 11.30 $\mu\text{g/g}$ for the fifth-instar caterpillar and adult butterfly, respectively.

Sensitivity of life-stage

Life stage of zebra longwing and white peacock did not affect the toxicity of permethrin. The 24-h LD50 values of zebra longwing fifth-instar caterpillars (0.11 $\mu\text{g/g}$) and adult butterfly (0.18 $\mu\text{g/g}$) were similar (Table 2). The 24-h LD50 values of white peacock fifth-instar caterpillars (0.79 $\mu\text{g/g}$) and adult butterflies (0.74 $\mu\text{g/g}$) were similar as well (Table 2). However, for atala hairstreak, common buckeye, and painted lady, the fifth-instar caterpillars were more sensitive to permethrin than were adult butterflies. The 24-h LD50 values of the fifth-instar caterpillars for permethrin were approximately 20, five, and two times lower than the 24-h LD 50 values of adult butterflies for atala hairstreak, common buckeye, and painted lady, respectively (Table 2).

In contrast to permethrin, life stage of zebra longwing, common buckeye, painted lady, and white peacock affected

the toxicity of naled. The fifth-instar caterpillars were more sensitive to naled than their adult butterflies. The 24-h LD50 values of the fifth-instar caterpillars were two, 1.7, three, and 77 times lower than the 24-h LD50 values of adult butterflies for zebra longwing, common buckeye, painted lady, and white peacock, respectively (Table 2).

For dichlorvos, life-stage of atala hairstreak and common buckeye affected the toxicity of dichlorvos. For example, atala hairstreak and common buckeye fifth-instar caterpillars were more sensitive to dichlorvos than their adult butterflies. The 24-h LD50 values of fifth-instar caterpillars were four and 1.5 times lower than the 24-h LD50 values of adult butterflies for atala hairstreak and common buckeye, respectively (Table 2). For zebra longwing, painted lady, and white peacock, the LD50s for fifth-instar caterpillars and adult butterflies were similar.

In general, fifth-instar caterpillars were slightly more sensitive to the adult mosquito control insecticides than were adult butterflies, but this was not significant, and exceptions occur.

Toxicity of insecticides—wing exposures

Although exposure of adult butterfly wings to insecticides was assumed not to produce biological effects, this study showed that exposure of adult butterfly forewings produced toxicological effects, as did exposures of the thorax. The adverse effects that occurred after exposure of the wings is attributable to absorption of toxicants into the veins, which function in oxygen and blood circulation. For all five adult species and wing exposure, the 24-h LD50 values for permethrin, naled, and dichlorvos ranged from 0.66 to 8.69 $\mu\text{g/g}$, 1.31 to 13.6 $\mu\text{g/g}$, and 1.34 to 6.68 $\mu\text{g/g}$, respectively (Table 3). For common buckeye, painted lady, and white peacock with permethrin, wing exposure resulted in higher 24-h LD50s than thorax exposures. However, with atala hairstreak, permethrin wing exposure resulted in lower LD50s than thorax exposure. With naled, atala hairstreak, painted lady, and white peacock were more sensitive to wing exposures than to thorax exposures. The LD50 concentrations as a result of naled wing exposures to atala hairstreak, painted lady, and white peacock were approximately four, 13, and 22 times lower, respectively, than those attributable to naled thorax exposure. However, common buckeye responded differently to naled. Wing exposure was less toxic than thorax exposure. For dichlorvos, wing exposures resulted in higher toxicity to atala hairstreak and common buckeye than thorax exposures. White peacock appeared to

be more sensitive to dichlorvos thorax exposures than to wing exposures. Dichlorvos toxicity was similar to that for painted lady and zebra longwing, regardless of exposure methods.

Toxicity: Comparison of butterflies with honeybees

Permethrin was demonstrated to be more toxic than naled, dichlorvos, and malathion to honeybees. With the honeybee, the LD50 for permethrin (0.024 $\mu\text{g/bee}$; [11]) was approximately 20 times lower than the naled LD50 (0.48 $\mu\text{g/bee}$; [5]) and dichlorvos LD50 (0.49 $\mu\text{g/bee}$; [43]) and 10 times lower than the malathion LD50 (0.2 $\mu\text{g/bee}$; [32]). A comparison of the LD50s for butterflies and insecticides on a μg insecticide/organism (thorax exposure) from this study (Table 2) with the honeybee LD50s indicates that, for permethrin and atala hairstreak (fifth instar) and zebra longwing (fifth instar and adult), LD50s are similar to those for honeybees; for naled, zebra longwing (fifth instar and adult) are more sensitive than honeybees; and for dichlorvos, zebra longwing and white peacock butterflies are more sensitive than honeybees. A comparison of the LD50s for butterflies and insecticides on a μg insecticide/organism (wing exposure) from this study (Table 3) with the honeybee LD50s indicates that, for permethrin, honeybees are more sensitive than butterflies; for naled, atala hairstreak and white peacock are more sensitive than honeybees; and for dichlorvos, atala hairstreak and zebra longwing are more sensitive than honeybees. In addition, based on the acute contact toxicity test hazard categorization scheme of Atkins et al. [43] for 24-h LD50s on a $\mu\text{g/organism}$ basis, all three insecticides are highly toxic to fifth-instar caterpillars and adult butterflies [43].

Based on these $\mu\text{g/bee}$ LD50s and the average weight of worker honeybees of 81 to 151 mg [44], the honeybee LD50s would be 3.19 to 5.93 $\mu\text{g/g}$ for naled, 3.28 to 6.1 $\mu\text{g/g}$ for dichlorvos, and 0.16 to 0.30 $\mu\text{g/g}$ for permethrin. Based on a comparison of larval and adult butterfly acute toxicities (from thorax exposure on a $\mu\text{g/g}$ basis) from this study, several butterfly species appear to have lower LD50s (e.g., fifth-instar atala hairstreak to permethrin) than honeybees to these insecticides, and therefore they may be more sensitive.

Importance of unit base

Results of lethal effect doses in micrograms per square meter are presented in Tables 2 and 3. With this unit base, the SSDs of adult butterfly LD50s for each insecticide (using both thorax

Table 3. Acute toxicity of permethrin, naled, and dichlorvos to Florida, USA, native butterflies as a result of exposure on the forewings^{a,b}

Compounds	Species	24-h LD50 ($\mu\text{g/g}$)	24-h LD50 ($\mu\text{g/m}^2$)	24-h LD50 ($\mu\text{g/organism}$)
Permethrin	Atala hairstreak	0.66 (0.46–0.95)	109 (76–157)	0.07 (0.05–0.10)
	Zebra longwing	— ^c	— ^c	— ^c
	Common buckeye	5.15 (3.87–6.84)	847 (638–1125)	0.72 (0.54–0.96)
	Painted lady	8.69 (7.29–10.36)	1838 (1538–2187)	1.76 (1.48–2.10)
	White peacock	2.55 (1.83–3.56)	262 (188–366)	0.26 (0.19–0.36)
Naled	Atala hairstreak	1.31 (1.06–1.62)	576 (466–712)	0.37 (0.30–0.46)
	Zebra longwing	— ^c	— ^c	— ^c
	Common buckeye	13.6 (10.5–17.6)	2338 (1,810–3,031)	2.00 (1.55–2.59)
	Painted lady	2.29 (1.93–2.72)	466 (393–554)	0.45 (0.38–0.53)
	White peacock	1.58 (1.18–2.13)	130 (97–175)	0.13 (0.01–0.17)
Dichlorvos	Atala hairstreak	1.73 (1.44–2.37)	357 (283–447)	0.23 (0.18–0.29)
	Zebra longwing	1.34 (0.89–2.05)	95 (63–145)	0.12 (0.08–0.19)
	Common buckeye	5.99 (4.06–8.82)	939 (637–1384)	0.80 (0.54–1.18)
	Painted lady	6.68 (4.11–10.84)	1474 (906–2391)	1.41 (0.87–2.30)
	White peacock	2.77 (2.02–3.78)	264 (193–361)	2.60 (0.19–0.36)

^a LD50 = median lethal dose.

^b Data in parentheses are 95% confidence interval.

^c Not studied.

and wing exposures) were plotted (Fig. 3). Permethrin had the lowest 10th percentile ($22.5 \mu\text{g}/\text{m}^2$) followed by naled ($85 \mu\text{g}/\text{m}^2$) and dichlorvos ($91.2 \mu\text{g}/\text{m}^2$). The permethrin SSD (Fig. 3A) in micrograms per square meter increased in order of atala hairstreak, painted lady, common buckeye, white peacock, and zebra longwing. This was similar to the sensitivity distribution of butterflies with microgram per gram unit base. Similarly, the sensitivity of butterflies to naled (Fig. 3b) was the same for both unit bases. However, for dichlorvos (Fig. 3c), the sensitivity of atala hairstreak and common buckeye was different for each unit base. With microgram per gram unit base, atala hairstreak was more sensitive to dichlorvos than common buckeye; however, with microgram per square meter unit base, atala hairstreak appeared to be less sensitive to dichlorvos than

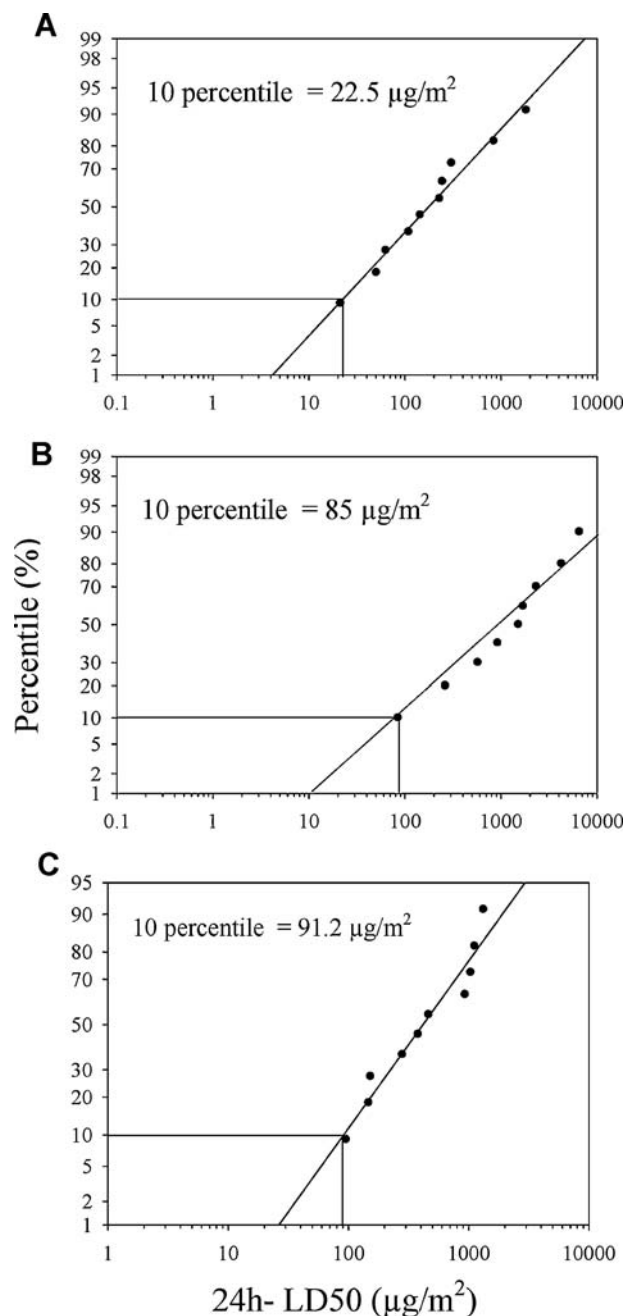


Fig. 3. Toxicity distribution of mosquito insecticides to five Florida, USA, native butterflies as a result of exposures on the thorax and forewings to permethrin (A), naled (B), and dichlorvos (C). LD50 = median lethal dose.

common buckeye. The total body surface area of atala hairstreak was smaller than the total body surface area of common buckeye. This would explain the discrepancy of the sensitivity of these two species to dichlorvos for different unit bases. In general, adult zebra longwing was the most sensitive butterfly as regards thorax exposure to all three insecticides. Adult zebra longwing was the most sensitive butterfly to wing exposure to dichlorvos, but it was not tested against permethrin and naled because we did not have stock culture available.

Hazard quotients

For permethrin, after field application, the highest concentration on filters was $80.35 \mu\text{g}/\text{m}^2$; however, on foliage it was $280.50 \mu\text{g}/\text{m}^2$ [38]. Therefore, the hazard quotients (HQs) for permethrin, using these environmental exposures and the lowest LD50s (Table 2) or 10th percentile ($22.5 \mu\text{g}/\text{m}^2$) indicates hazard to most adult butterfly species tested in the current study. For naled, the highest concentrations on filters of $1,373.08 \mu\text{g}/\text{m}^2$ [39] and $74.85 \mu\text{g}/\text{m}^2$ [38] and on foliage of $215.53 \mu\text{g}/\text{m}^2$ [38] along with acute toxicity data and the SSD also indicate hazard of naled (e.g., zebra longwing) to adult butterfly species. The latter HQ results for naled become more significant because significant butterfly mortality (40–100%) occurred in drift and direct application areas after actual use of naled [39]. For dichlorvos, the highest concentration on foliage of $41.61 \mu\text{g}/\text{m}^2$ [38] after application of naled indicates no hazard of dichlorvos to any adult butterflies using the existing acute toxicity data and 10th percentile from the SSD for dichlorvos.

Testing of nontarget insects

As previously mentioned, the honeybee (*Apis mellifera* L.) has been the beneficial, nontarget insect test species required for laboratory toxicity testing by chemical manufacturers of pesticides under the FIFRA by the U.S. EPA. The selection of the honeybee in toxicity testing results from its key role in agriculture of supplying approximately 85% of the pollination necessary to provide one third of our food supply. Three primary routes of exposure can be seen for bees after pesticide application in the field: through direct spray, through uptake of nectar or pollen, and through contact with treated foliage or flowers [31].

A variety of laboratory toxicity methods have evolved for testing honeybees [37–41] and bumblebees (*Bombus* sp.; e.g., *B. terrestris*; Apidae) [31,45,46] to pesticides via different routes of exposure: contact directly through topical application and indirectly through pesticide-treated foliage and feeding orally. The comparative laboratory honeybee toxicity studies conducted by Atkins et al. since the 1950s at the University of California (Riverside) set the foundation and framework for much of what has been done today on the evaluation of pesticide toxicity to honeybees [43,47,48]. Atkins et al. also developed an acute contact toxicity test hazard categorization scheme based on contact LD50 results (e.g., $<2 \mu\text{g a.i./bee}$ = highly toxic; 2 to $<11 \mu\text{g a.i./bee}$ = moderately toxic; $\geq 11 \mu\text{g a.i./bee}$ = practically nontoxic) [43].

Three standard types of honeybee laboratory toxicity tests may be conducted according to validated methods: acute contact LD50 [32], acute oral LD50 [34], and acute contact with treated foliage [33]. The tests have focused on acute exposure (24–48 h) and death as the biological endpoint. The exposure quantity producing the LD50 is reported as the field application rate (lb a.i./ac) or the quantity directly applied to the bee ($\mu\text{g a.i./bee}$). The honeybee standard methods for U.S. EPA suggest

using young bees and the Organization for Economic Cooperation and Development suggest using young adult worker bees. Most bee toxicity test standards in the United States have no specific test requirement as to the type (worker, drone, or queen) or stage (egg, larvae, pupa, adult) to use.

With more than 30 orders, 125 families, and approximately one million described insect species, using the honeybee as the surrogate to represent all members within this taxa is clearly an underestimate to assess potential effects of contaminants on beneficial insects, given the unique roles of insects in the ecosystem and their potential for extinction [49]. In addition, in view of the differences in pesticide modes of action, it is likely that other insect species are more sensitive than honeybees. The honeybee is also not representative of insects that have large surface area per volume ratio, such as butterflies, which are more likely to encounter higher exposure concentrations in the field than are honeybees. In addition, in honeybee toxicity testing, application of the pesticide is to part of the thorax of the bee, which is only partially representative of actual field exposures, in which the pesticide is likely to be distributed over a greater part of the insect body as a result of drift. Furthermore, different insect life stages (e.g., larvae vs adult), as in other nontarget organism toxicity testing, may respond dramatically differently to pesticide exposures and is currently not considered in honeybee toxicity testing.

CONCLUSIONS

Honeybees are the major pollinator of food crops and the standard insect test species used for toxicity testing of pesticides on nontarget insects under FIFRA. Adult mosquito control insecticides are highly toxic to honeybees, based on acute exposure. The current study conducted acute toxicity tests with adult mosquito control insecticides (naled, permethrin) and the metabolite dichlorvos on fifth larval instar (caterpillars) and adults of different native Florida butterfly species to determine 24-h LD50s, because limited acute toxicity data are available with this major insect group of pollinators. Thorax- and wing-only applications of each insecticide were conducted. Based on LD50s, thorax and wing application exposures were acutely toxic to both caterpillars and adults. Permethrin was the most acutely toxic insecticide after thorax exposure to fifth instars and adult butterflies. A broad range of acute toxicities was seen between species. However, no generalization on acute toxicity (sensitivity) of the insecticides could be concluded based on exposures to fifth instars versus adult butterflies or on thorax versus wing exposures of adult butterflies.

A comparison of LD50s of the butterflies from this study (caterpillars and adults) with honeybee LD50s for the adult mosquito insecticides, on a microgram per organism or microgram per gram basis, indicates that several butterfly species are more sensitive to these insecticides than are honeybees. Using an HQ approach (or the ratio of the highest actual environmental exposure after application to the lowest LD50 or the 10th percentile of the SSD from this study) indicates that both permethrin and naled applications in the field may present potential acute hazards to butterflies, whereas no acute hazard of dichlorvos to butterflies is seen. Although no well-designed field study of butterflies and permethrin application exists, an ecological incident report by U.S. EPA of butterfly mortalities associated with actual application of permethrin in the field has been published. The acute hazard of naled to butterflies in south Florida, however, is supported by an actual field application study of a single application of naled, with significant butterfly

mortalities following the exposure. Hazards to butterflies in the field to all three insecticides may be exacerbated by increased frequency of use of mosquito control insecticides, within short time durations, which may occur during periods of high mosquito pressure.

Although the cost of culturing or purchasing butterflies is higher than that for honeybees, and testing is labor intensive, butterflies are a valued ecological resource that should be considered as potential test organisms when nontarget insect testing of pesticides is suggested under FIFRA.

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