

Article

Toxicity of azadirachtin against larval instars of *Spodoptera frugiperda* (J.E. Smith, 1797): ontogenetic thresholds and lethal time dynamics under high-mountain laboratory conditions

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Abstract

Azadirachtin, derived from *Azadirachta indica* A. Juss., is a botanical insecticide of growing relevance for Integrated Pest Management (IPM). Most toxicological data on azadirachtin against *Spodoptera frugiperda* (J.E. Smith, 1797) have been generated under lowland tropical conditions (20-30°C), leaving a critical knowledge gap regarding its efficacy in Andean high-mountain agroecosystems. Dose-response and lethal time bioassays against all six larval instars were conducted at 2 586 m a.s.l. (17 ± 1°C, 65 ± 10% RH). Early instars (L1-L3) showed significantly higher susceptibility, with LC₅₀ values below 703 µL/L at 96 h and mean LT₅₀ of 43.3 h. A critical physiological threshold between L3 and L4 was identified through Probit analysis (R² = 0.986, p < 0.001) and lethal time curves, with LT₅₀ increasing 2.7-fold from L3 to L4 (37.9 vs. 145.9 h). Three-way ANOVA confirmed that larval instar (partial η² = 0.399, p < 0.001) and exposure time (partial η² = 0.355, p < 0.001) were the dominant factors determining mortality. Azadirachtin retains biologically significant activity against *S. frugiperda* under high-mountain conditions, but its efficacy is strongly dependent on larval ontogeny. The L3-L4 threshold represents an actionable decision point for IPM: treatments should target L1-L3 instars to maximize cost-effectiveness and minimize the risk of inconsistent field outcomes.

Additional keywords: nazadirachtin, botanical insecticides, fall armyworm, high-altitude agroecosystem, integrated pest management, neem oil

Resumen

La azadirachtina, derivada de *Azadirachta indica* A. Juss., es un insecticida botánico de creciente relevancia para programas de Manejo Integrado de Plagas (MIP). La mayoría de los datos toxicológicos sobre azadirachtina frente a *Spodoptera frugiperda* (J.E. Smith, 1797) han sido generados en condiciones de tierras bajas tropicales (20-30°C), dejando un vacío de conocimiento sobre su eficacia en agroecosistemas de alta montaña andina. Se realizaron bioensayos de dosis-respuesta y tiempo letal contra los seis estadios larvales a 2 586 m s.n.m. (17 ± 1°C, 65 ± 10% HR). Los estadios tempranos (L1-L3) mostraron mayor susceptibilidad, con valores de CL₅₀ inferiores a 703 µL/L a las 96 h y un TL₅₀ medio de 43,3 h. Se identificó un umbral fisiológico crítico entre L3 y L4, con el TL₅₀ aumentando 2,7 veces de L3 a L4 (37,9 vs. 145,9 h). Un ANOVA trifactorial confirmó que el estadio larval (η² parcial = 0,399, p < 0,001) y el tiempo de exposición (η² parcial = 0,355, p < 0,001) fueron los factores dominantes. La azadirachtina mantiene actividad insecticida biológicamente significativa contra *S. frugiperda* bajo condiciones de alta montaña, siendo su eficacia fundamentalmente dependiente de la ontogenia larval. El umbral L3-L4 constituye un punto de decisión accionable para el MIP: las aplicaciones deben dirigirse a los estadios L1-L3 para maximizar la rentabilidad.

Palabras clave: fazadirachtina, agroecosistema de alta montaña, gusano cogollero, insecticidas botánicos, manejo integrado de plagas, aceite de neem

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Introduction

Spodoptera frugiperda (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is one of the most economically destructive pests of maize throughout the Neotropics, capable of inflicting crop losses ranging from 20 to 100% when populations are not managed effectively (Tay et al. 2023). Native to the Americas, the species has demonstrated remarkable adaptive capacity, invading sub-Saharan Africa in 2016 and subsequently spreading across Asia and Oceania, where it now threatens food security at a continental scale (Kenis et al. 2023). Its high reproductive rate, polyphagous feeding behavior, and documented capacity to develop resistance to synthetic insecticides have intensified interest in sustainable alternatives compatible with Integrated Pest Management (IPM) frameworks (Abbas et al. 2022).

Botanical insecticides derived from *Azadirachta indica* A. Juss. (Meliaceae) represent one of the most thoroughly studied biopesticide options for *S. frugiperda* management. Azadirachtin, the principal bioactive limonoid in neem seed extracts, operates through a multi-modal mechanism: it acts simultaneously as an antifeedant, an insect growth regulator (IGR), and an endocrine disruptor (Isman 2006). At the molecular level, azadirachtin interferes with the neuroendocrine cascade regulating ecdysone biosynthesis and release, blocking the production of prothoracicotropic hormone (PTTH) and suppressing 20-hydroxyecdysone titers required for normal molting (Mordue and Nisbet 2000). Recent studies have demonstrated that azadirachtin inhibits the nuclear receptor HR3 in the prothoracic gland, thereby blocking larval ecdysis in *S. frugiperda* at the molecular level (Fan et al. 2023). These mechanisms result in larval mortality through starvation, failed molting, and developmental arrest, effects most pronounced in early instars that depend heavily on rapid hormonal cycling. Despite a large body of published bioassay data on neem against *S. frugiperda*, a critical environmental variable has received little attention: the role of temperature in modulating azadirachtin efficacy. Virtually all published LC_{50} and LT_{50} estimates for this species have been generated under lowland tropical conditions (20–30°C), which do not represent the thermal regime of Andean maize production systems. High-altitude agroecosystems in the Colombian Andes commonly experience

temperatures of 14–18°C, conditions known to reduce insect metabolic rates, extend developmental periods, and potentially alter the uptake and detoxification of ingested compounds (Yan et al. 2022). Direct evidence indicates that azadirachtin toxicity against Orthoptera and Lepidoptera is highly temperature-sensitive, with efficacy declining substantially at lower temperatures (Kabaru and Mwangi 2000). If published dose recommendations derived from lowland bioassays are applied uncorrected in highland systems, growers risk suboptimal control outcomes or unnecessarily high concentrations.

A secondary knowledge gap concerns instar-specific susceptibility. Although reduced sensitivity of later instars to both synthetic and botanical insecticides has been widely noted in *S. frugiperda* (Carvalho et al. 2013, Yu 2015), few studies have combined LC_{50} , LT_{50} , and time-mortality analyses across all six instars to precisely localize the ontogenetic threshold at which efficacy decreases sharply. Such precision is practically important because maize whorl monitoring rarely records first-instar larvae; action thresholds are typically based on the presence of L2–L4 larvae, making the exact position of the susceptibility boundary critical information for timing interventions.

The present study addresses both gaps. Specifically, we: (i) characterize dose-response and lethal time relationships for azadirachtin against all six larval instars of *S. frugiperda* under high-mountain laboratory conditions (2 586 m a.s.l., 17°C); (ii) identify and quantify the ontogenetic threshold between susceptible and tolerant instars; and (iii) assess the main and interactive effects of instar, concentration, and exposure time on larval mortality using a trifactorial ANOVA. The results provide the first comprehensive toxicological baseline for azadirachtin in Andean highland environments and offer actionable guidelines for neem-based IPM in high-altitude maize.

Materials and methods

Insect colony and larval rearing

Larvae of *S. frugiperda* were obtained from a colony maintained at the Agronomic Engineering Laboratory of the Universidad de Pamplona (Pamplona, Norte de Santander, Colombia; lat 7°22'02" N, long 72°38'56" W; 2 586 m a.s.l.). The colony was established from field-collected individuals and reared under standardized conditions (17 ± 1°C, 65 ± 10% RH, 12:12 h L:D photoperiod) on a semi-

artificial maize-based diet (Giraldo-Vanegas et al. 2026). Mean developmental durations for each larval instar are summarized in Table 1. The prolonged larval period, particularly the sixth instar (mean 13.63 ± 1.75 days), reflects developmental slowing in cold-adapted insect populations at high altitude (Zanzana et al. 2024). All larvae used in bioassays were selected at precise ages within each instar to minimize overlap between consecutive developmental stages (Table 1).

Test insecticide

The commercial formulation NEEM-X 1.2% EC (*Azadirachta indica* A. Juss. seed extract; 1.2% w/v azadirachtin equivalent, 12 g active ingredient per liter) was used throughout the study. Concentrations of technical-grade azadirachtin (mg/L) were calculated from the commercial formulation as described in Table 2. Seven concentrations, including an absolute control, were evaluated: 0, 275, 550, 833, 1 108, 1 383, and 1 667 $\mu\text{L/L}$ of commercial product, equivalent to 0 to 20.00 mg/L of technical azadirachtin.

Bioassay conditions and design

Dose-response bioassays were conducted by immersing fresh maize leaf sections (5 cm²) for 30 s in each test concentration, air-drying for 60 min at room temperature, and offering them to individual larvae confined in plastic Petri dishes (9 cm diameter) lined with moist filter paper. One larva per dish was used to avoid confounding effects of larval crowding and cannibalism. Twenty-five larvae per concentration per instar were tested for each of the seven concentrations, yielding 175 experimental units per instar and 1 050 total units across all six instars. Absolute controls were prepared by immersing leaf

sections in distilled water supplemented with the same volume of carrier solvent used in the treatments.

Mortality was recorded at 12, 24, 48, 72, and 96 h after initial exposure. A larva was scored as dead when it showed no response to gentle mechanical stimulation with a fine brush. Abbott's correction formula was applied to all mortality values to account for natural mortality in control treatments. Control mortality remained below acceptable thresholds throughout the experiment (mean 0.66%, maximum 4.00% in L6 controls), confirming bioassay validity (Table 3).

Statistical analyses

Dose-response data at 96 h were analyzed by Probit regression (Finney 1971) to estimate the median lethal concentration (LC_{50}) and its 95% confidence interval (CI) for each larval instar. An integrated model combining all instars was also fitted. Lethal time (LT_{50}) values were estimated by fitting independent Probit regression equations to the time-mortality data at each concentration for each instar. Goodness of fit was assessed by the coefficient of determination (R^2). Linear regression was used to test the significance of the relationship between instar number and LC_{50} values ($R^2 = 0.986$, $p < 0.001$). A three-way analysis of variance (ANOVA) was conducted to evaluate the independent and interactive effects of larval instar (6 levels), neem oil concentration (6 treatment levels, excluding control), and exposure time (8 recording intervals) on larval mortality, using Type III sum of squares and partial η^2 as effect size measure. Post hoc comparisons were performed using Tukey's HSD test ($\alpha = 0.05$). All analyses were performed using SPSS v.26.0 (IBM Corp., Armonk, NY, USA).

Table 1. Developmental parameters of larval instars of *S. frugiperda* under laboratory conditions ($17 \pm 1^\circ\text{C}$, $65 \pm 10\%$ RH) and selected ages for bioassays.

Instar	Mean duration (days $\pm$ SD)	Cumulative age (days)	Selected age for bioassay (days)
L1	5.19 ± 0.39	5.19	3 ± 0.5
L2	3.67 ± 0.53	8.86	7 ± 0.5
L3	4.36 ± 0.54	13.22	11 ± 0.5
L4	5.93 ± 0.46	19.15	17 ± 0.5
L5	6.39 ± 1.22	25.54	22 ± 0.5
L6	13.63 ± 1.75	39.17	35 ± 1.0

Note: Mean developmental durations adapted from Giraldo-Vanegas et al. (2026). Selected ages were defined to minimize overlap between consecutive instars given natural developmental variability.

Table 2. Azadirachtin (neem oil 1.2% EC) concentrations evaluated in the toxicological bioassay against *S. frugiperda*.

Treatment	Commercial product concentration (mL/L)	Applied azadirachtin ($\mu\text{L/L}$)	Technical azadirachtin (mg/L)
T1 (Control)	0.00	0	0.00
T2	0.33	275	3.30
T3	0.66	550	6.60
T4	1.00	833	10.00
T5	1.33	1 108	13.30
T6	1.66	1 383	16.60
T7	2.00	1 667	20.00

Table 3. Mortality recorded in absolute controls by larval instar of *S. frugiperda*.

Instar	Control mortality (%)	n (evaluated larvae)
L1	0.00	25
L2	0.00	25
L3	0.00	25
L4	0.00	25
L5	0.50	25
L6	4.00	25
Mean	0.66	150

Results

Control mortality and general dose-mortality response

Control-corrected mortality remained consistently low across all six instars (mean 0.66%; range 0.00-4.00%), confirming bioassay reliability (Table 3). The integrated Probit model combining all instars yielded a robust concentration-mortality relationship ($R^2 = 0.89$), with a general LC_{50} of 740.4 $\mu\text{L/L}$ of commercial product (equivalent to 8.88 mg/L technical azadirachtin) (Figure 1). Mortality increased sharply at concentrations above 275 $\mu\text{L/L}$, with all treatments from 275 $\mu\text{L/L}$ onward reaching 100% mortality in L1-L3 at 96 h and near-total mortality ($\geq 99.2\%$) in all instars (Table 4).

Instar-specific LC_{50} and ontogenetic threshold

Probit analyses revealed a highly significant and near-linear increase in LC_{50} values from L1 to L6 ($R^2 = 0.986$, $p < 0.001$; Table 5, Figure 2). First-instar larvae were the most susceptible ($\text{LC}_{50} = 579.7 \mu\text{L/L}$; 95% CI: 512.3-655.2), whereas sixth-instar larvae required a 59% higher

concentration ($\text{LC}_{50} = 921.4 \mu\text{L/L}$; 95% CI: 835.1-1 015.7) to achieve 50% mortality at 96 h. Despite the continuous increase in LC_{50} across instars, the critical increase in resistance occurred at the L3-L4 transition: the proportional increase in LC_{50} from L3 to L4 (13.2%) was equivalent to the cumulative increase observed across L1-L3 (13.2%), indicating an abrupt acceleration of the resistance gradient at this developmental point. Probit regression slopes were consistent across instars (range: 3.14-3.28), indicating similar dose-response steepness regardless of instar.

Lethal time (LT_{50}) dynamics and the L3-L4 physiological threshold

LT_{50} analyses revealed an even more pronounced ontogenetic discontinuity than LC_{50} data (Tables 6-11, Figure 3). Mean LT_{50} across all concentrations for early instars (L1-L3) was 43.3 ± 12.6 h, compared to 159.5 ± 70.6 h for late instars (L4-L6), a 3.7-fold difference. The transition between L3 and L4 produced the single largest discrete jump in the dataset: mean LT_{50} increased from 37.9

h (L3) to 145.9 h (L4), a 2.7-fold (271.3%) increase within a single instar transition. This inflection point constitutes a critical physiological threshold in larval susceptibility to azadirachtin under high-mountain conditions.

Time-mortality curves further illustrated this pattern (Figure 4). For L1-L3, steep sigmoidal mortality curves were observed, with 50% larval mortality achieved within 21-67 h across concentrations. For L4-L6, mortality curves were markedly flattened and shifted to the right, with LT_{50} values ranging from 91 to 410 h. Even at the highest concentration tested (1 667 $\mu\text{L/L}$), L5 larvae required a median time of 95.2 h to reach 50% mortality. The R^2 values for LT_{50} regression equations were generally lower for late instars (0.12-0.57) compared to early instars (0.55-0.78), reflecting greater variability in the time course of mortality in older, more physiologically robust larvae.

Three-way ANOVA: contributions of instar, concentration, and time

The three-way ANOVA confirmed that all three main factors significantly influenced larval mortality (Table 12). Larval instar was the strongest determinant of mortality (partial $\eta^2 = 0.399$, $F_{5,1152} = 152.70$, $p < 0.001$), followed by exposure time (partial $\eta^2 = 0.355$, $F_{7,1152} = 90.44$, $p < 0.001$) and concentration (partial $\eta^2 = 0.049$, $F_{5,1152} = 11.79$, $p < 0.001$). Two-way interactions between instar $\times$ concentration (partial $\eta^2 = 0.063$, $p < 0.001$) and instar $\times$ time (partial $\eta^2 = 0.087$, $p < 0.001$) were both significant, indicating that the effects of dose and exposure time on mortality are not independent of larval developmental stage. The interaction between concentration $\times$ time was non-significant ($p = 0.249$). The three-way interaction (instar $\times$ concentration $\times$ time) was also non-significant ($p = 0.953$), consistent with a model in which developmental stage is the primary modulator of azadirachtin efficacy.

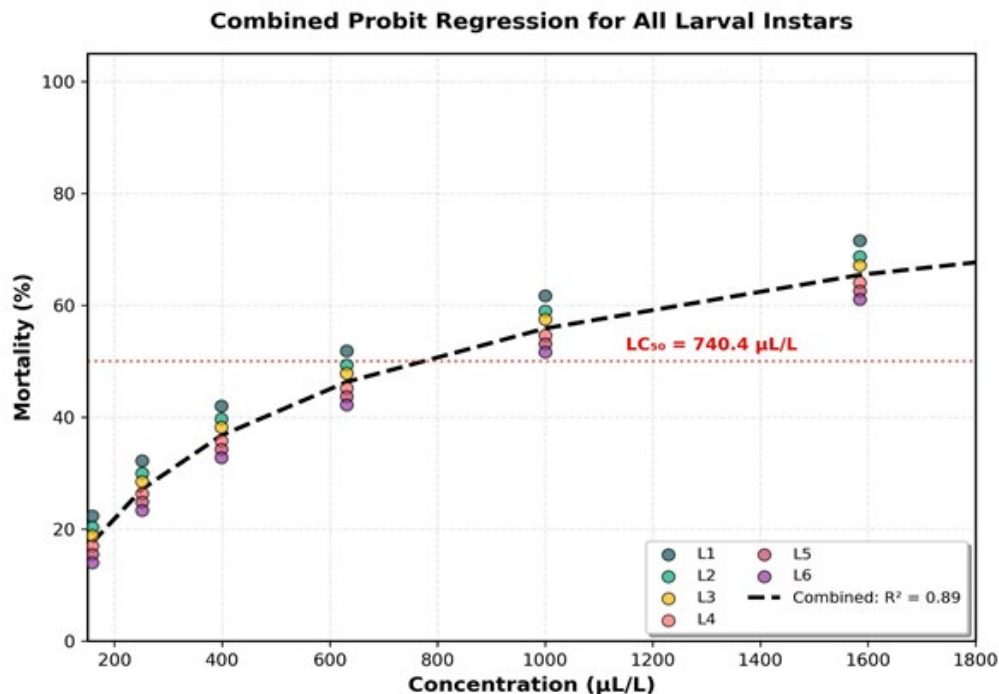


Figure 1. Combined Probit regression for all larval instars of *S. frugiperda*, exposed to azadirachtin under high-mountain laboratory conditions (2 586 m a.s.l.). The red dotted line indicates 50% mortality. The general $LC_{50} = 740.4 \mu\text{L/L}$ ($R^2 = 0.89$) is indicated. Points are color-coded by larval instar (L1-L6).

Table 4. Total mortality (%) caused by different neem oil (azadirachtin 1.2% EC) concentrations on six larval instars of *S. frugiperda* at 96 h of exposure.

Concentration ($\mu\text{L/L}$)	Mean (%)	L1 (%)	L2 (%)	L3 (%)	L4 (%)	L5 (%)	L6 (%)
0 (Control)	0.66 a	0.00 a	0.00 a	0.00 a	0.00 a	0.50 a	4.00 a
275	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b
550	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b
833	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b
1 108	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b
1 383	99.87 b	99.20 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b
1 667	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b	100.00 b

Note: Values followed by different letters within each column are significantly different according to Tukey's HSD test ($p \leq 0.05$). $n = 25$ larvae per treatment-instar combination.

Table 5. Probit regression equations, coefficients of determination (R^2), and median lethal concentration (LC_{50}) of azadirachtin (neem oil 1.2% EC) for each larval instar of *S. frugiperda* at 96 h of exposure.

Instar	Probit regression equation	R^2	$\log_{10}$ (LC_{50})	LC_{50} ($\mu\text{L/L}$)	95% CI ($\mu\text{L/L}$)
L1	$Y = 3.28X - 4.06$	0.95	2.76	579.7	512.3-655.2
L2	$Y = 3.22X - 4.06$	0.95	2.82	654.2	585.1-731.4
L3	$Y = 3.22X - 4.16$	0.95	2.85	702.7	631.8-782.1
L4	$Y = 3.14X - 4.11$	0.94	2.90	795.7	717.4-882.5
L5	$Y = 3.14X - 4.21$	0.94	2.93	856.2	774.2-946.8
L6	$Y = 3.14X - 4.31$	0.94	2.96	921.4	835.1-1 015.7

Note: Y = Probit (mortality); X = $\log_{10}$ (concentration in $\mu\text{L/L}$); 95% CI = 95% confidence interval. LC_{50} values in $\mu\text{L/L}$ of commercial product (1.2% EC formulation).

Discussion

This study demonstrates that azadirachtin retains biologically significant insecticidal and growth-regulatory activity against all larval instars of *S. frugiperda* under high-mountain laboratory conditions (17°C, 2 586 m a.s.l.), but that its efficacy is fundamentally modulated by larval ontogeny. The general LC_{50} of 740.4 $\mu\text{L/L}$ obtained in this study is consistent with the range reported in Brazilian studies under lowland conditions (600-1 200 $\mu\text{L/L}$ for similar commercial formulations) (Tulashie et al. 2021), suggesting that azadirachtin effectiveness in Andean highland systems is not substantially compromised by the cooler thermal regime, at least when targeting early instars. This partially contrasts with the classic finding that *A. indica* extracts exhibit markedly reduced toxicity

against other Orthoptera at temperatures below 22°C (Kabar and Mwangi 2000) and may reflect the fact that azadirachtin's primary mode of action, disruption of hormonal molting cascades rather than neural-mediated toxicity, is less temperature-dependent than neurotoxic insecticides.

The molecular basis for azadirachtin's efficacy against early instars is well-supported by recent mechanistic work. Fan et al. (2023) demonstrated that azadirachtin specifically inhibits the nuclear receptor HR3 in the prothoracic gland, blocking the ecdysone biosynthesis pathway and preventing larval ecdysis in *S. frugiperda*. This mechanism is most disruptive in early instars that are actively preparing for rapid molt cycles and whose prothoracic gland activity is at its peak (Mordue and Nisbet 2000). In contrast, fifth- and sixth-instar larvae

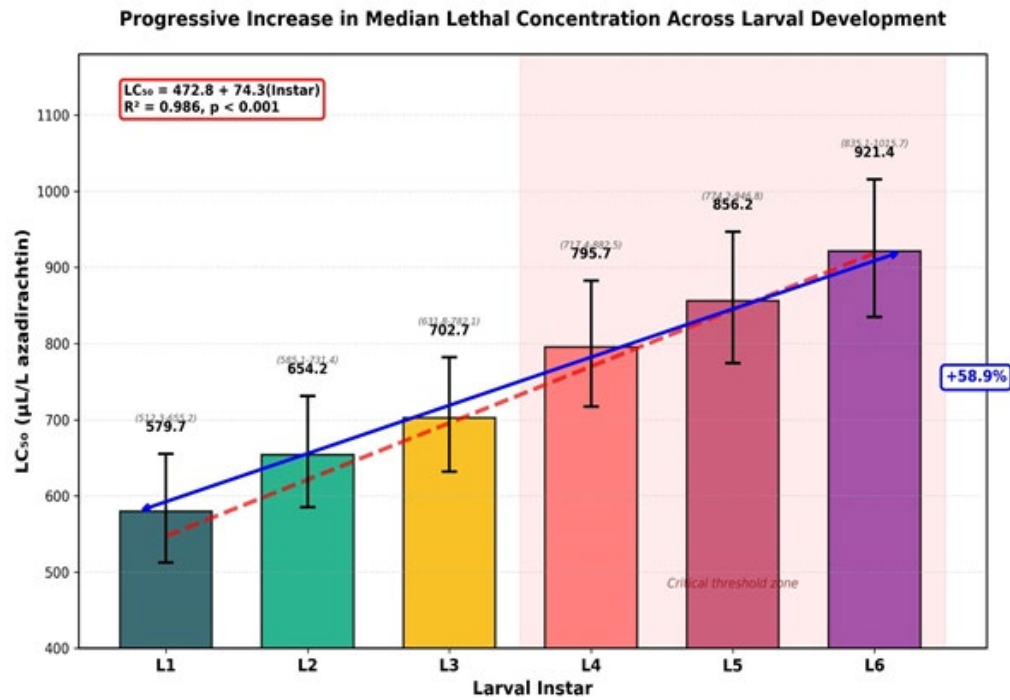


Figure 2. Progressive increase in median lethal concentration (LC₅₀) of azadirachtin across larval development of *S. frugiperda*. Error bars represent 95% confidence intervals. The shaded region (L4-L6) indicates the critical threshold zone of reduced susceptibility. The blue regression line shows the significant linear relationship ($R^2 = 0.986$, $p < 0.001$; $LC_{50} = 472.8 + 74.3 \times \text{instar}$). The total increase from L1 to L6 (+58.9%) is annotated.

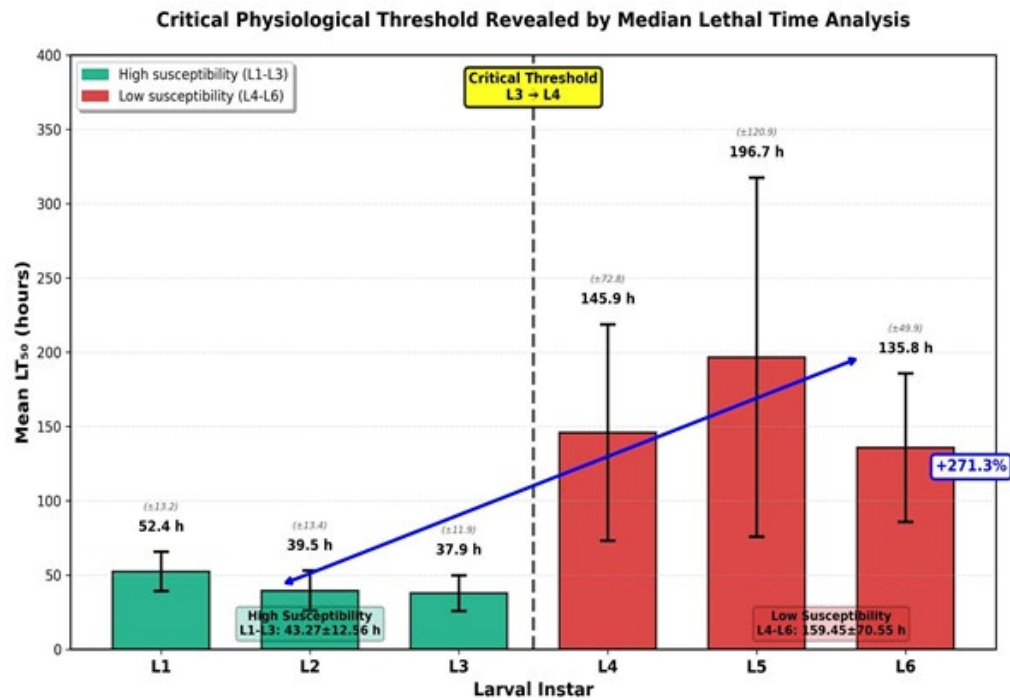


Figure 3. Critical physiological threshold revealed by mean LT₅₀ analysis across larval instars of *S. frugiperda*. Green bars represent early instars (L1-L3; mean LT₅₀ = 43.27 ± 12.56 h); red bars represent late instars (L4-L6; mean LT₅₀ = 159.45 ± 70.55 h). The dashed vertical line marks the L3→L4 threshold, corresponding to a 271.3% increase in mean LT₅₀. Error bars represent standard deviation across concentrations.

Table 6. Median lethal time (LT₅₀), Probit regression equations, and coefficients of determination (R²) for first-instar larvae of *S. frugiperda* exposed to different azadirachtin concentrations.

Concentration ($\mu\text{L/L}$)	Regression equation	R ²	LT ₅₀ (h)	95% CI (h)
275	$Y = 0.90X + 4.86$	0.71	50.16	43.2-57.8
550	$Y = 0.82X + 2.43$	0.55	58.01	48.9-68.2
833	$Y = 0.94X - 13.00$	0.77	67.02	59.1-75.8
1 108	$Y = 0.99X - 2.86$	0.78	53.39	46.8-60.5
1 383	$Y = 0.86X + 23.28$	0.60	31.07	24.1-39.2
1 667	$Y = 0.73X + 10.14$	0.65	54.60	46.9-63.1

Note: Y = Probit (mortality); X = time (hours); 95% CI = 95% confidence interval.

Table 7. Median lethal time (LT₅₀), Probit regression equations, and coefficients of determination (R²) for second-instar larvae of *S. frugiperda* exposed to different azadirachtin concentrations.

Concentration ($\mu\text{L/L}$)	Regression equation	R ²	LT ₅₀ (h)	95% CI (h)
275	$Y = 1.03X - 6.71$	0.74	54.99	48.3-62.1
550	$Y = 1.18X - 11.14$	0.74	51.81	45.7-58.4
833	$Y = 0.99X + 13.14$	0.67	37.23	31.2-44.1
1 108	$Y = 1.05X + 7.43$	0.74	40.54	35.1-46.8
1 383	$Y = 0.94X + 20.43$	0.74	31.46	26.4-37.2
1 667	$Y = 0.86X + 31.71$	0.61	21.27	16.8-26.5

Note: Y = Probit (mortality); X = time (hours); 95% CI = 95% confidence interval.

Table 8. Median lethal time (LT₅₀), Probit regression equations, and coefficients of determination (R²) for third-instar larvae of *S. frugiperda* exposed to different azadirachtin concentrations.

Concentration ($\mu\text{L/L}$)	Regression equation	R ²	LT ₅₀ (h)	95% CI (h)
275	$Y = 0.69X + 10.43$	0.75	57.35	49.8-65.7
550	$Y = 0.80X + 9.23$	0.63	50.96	43.1-59.8
833	$Y = 0.87X + 23.86$	0.64	30.05	24.2-36.8
1 108	$Y = 1.98X - 29.14$	0.13	39.97	28.6-55.2
1 383	$Y = 0.94X - 3.57$	0.68	57.00	49.5-65.3
1 667	$Y = 0.77X + 26.71$	0.50	30.25	23.1-38.9

Note: Y = Probit (mortality); X = time (hours); 95% CI = 95% confidence interval.

Table 9. Median lethal time (LT₅₀), Probit regression equations, and coefficients of determination (R²) for fourth-instar larvae of *S. frugiperda* exposed to different azadirachtin concentrations.

Concentration ($\mu\text{L/L}$)	Regression equation	R ²	LT ₅₀ (h)	95% CI (h)
275	$Y = 0.48X - 8.14$	0.47	121.13	98.7-148.2
550	$Y = 0.17X + 0.28$	0.12	292.48	215.3-396.8
833	$Y = 0.60X - 10.87$	0.43	101.45	82.1-125.4
1 108	$Y = 0.55X - 7.28$	0.33	104.15	84.2-128.9
1 383	$Y = 0.73X - 16.14$	0.57	90.60	76.8-106.9
1 667	$Y = 0.34X - 6.43$	0.56	165.97	139.2-197.8

Note: Y = Probit (mortality); X = time (hours); 95% CI = 95% confidence interval.

Table 10. Median lethal time (LT₅₀), Probit regression equations, and coefficients of determination (R²) for fifth-instar larvae of *S. frugiperda* exposed to different azadirachtin concentrations.

Concentration ($\mu\text{L/L}$)	Regression equation	R ²	LT ₅₀ (h)	95% CI (h)
275	$Y = 0.26X - 6.14$	0.38	215.92	165.3-282.1
550	$Y = 0.13X - 3.28$	0.16	409.85	289.7-579.4
833	$Y = 0.23X - 4.14$	0.23	235.39	176.2-314.8
1 108	$Y = 0.49X - 6.57$	0.46	115.45	95.8-139.2
1 383	$Y = 0.53X - 7.43$	0.48	108.36	90.3-130.1
1 667	$Y = 0.65X - 11.86$	0.48	95.17	81.2-111.5

Note: Y = Probit (mortality); X = time (hours); 95% CI = 95% confidence interval.

Table 11. Median lethal time (LT₅₀), Probit regression equations, and coefficients of determination (R²) for sixth-instar larvae of *S. frugiperda* exposed to different azadirachtin concentrations.

Concentration ($\mu\text{L/L}$)	Regression equation	R ²	LT ₅₀ (h)	95% CI (h)
275	$Y = 0.40X - 11.14$	0.47	152.85	128.7-181.5
550	$Y = 0.27X - 8.28$	0.34	215.85	172.4-270.3
833	$Y = 0.37X - 9.14$	0.38	159.84	133.2-191.9
1 108	$Y = 0.63X - 11.57$	0.55	97.73	84.5-113.0
1 383	$Y = 0.67X - 12.43$	0.57	93.18	81.2-106.9
1 667	$Y = 0.65X - 11.86$	0.48	95.17	81.9-110.5

Note: Y = Probit (mortality); X = time (hours); 95% CI = 95% confidence interval.

Time-Mortality Curves Across All Six Larval Instars: Progressive Reduction in Azadirachtin Action Speed

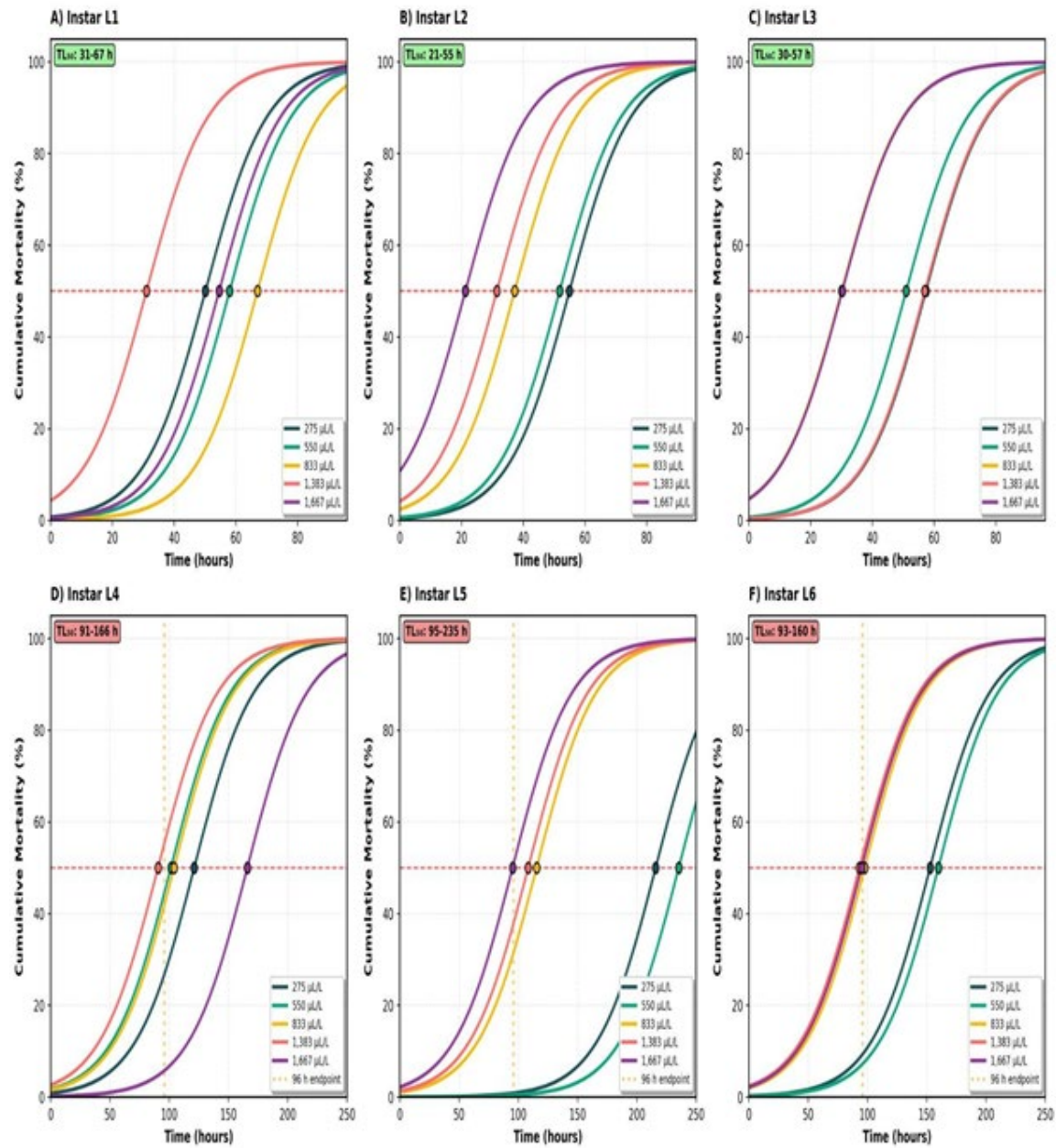


Figure 4. Time-mortality curves across all six larval instars of *S. frugiperda* exposed to azadirachtin: (A) L1, (B) L2, (C) L3, (D) L4, (E) L5, and (F) L6. Each curve represents a different azadirachtin concentration (275-1 667 $\mu\text{L/L}$). Note the progressive rightward shift and flattening of curves from early (L1-L3) to late (L4-L6) instars, and the expanded time scale for L4-L6 panels. LT50 ranges are annotated for each panel.

Table 12. Three-way analysis of variance (ANOVA) of the effects of larval instar, azadirachtin concentration, and exposure time on mortality of *S. frugiperda*.

Source of variation	Type III SS	df	MS	F	p-value	η^2p
Corrected model	1 584 261.12	287	5 520.07	6.36	<0.001	0.613
Instar	662 453.09	5	132 490.62	152.70	<0.001	0.399
Concentration	51 152.59	5	10 230.52	11.79	<0.001	0.049
Time	549 314.70	7	78 473.53	90.44	<0.001	0.355
Instar $\times$ Concentration	67 286.27	25	2 691.45	3.10	<0.001	0.063
Instar $\times$ Time	94 741.16	35	2 706.89	3.12	<0.001	0.087
Concentration $\times$ Time	35 029.66	35	1 000.85	1.15	0.249	0.034
Instar $\times$ Conc. $\times$ Time	124 283.65	175	710.19	0.82	0.953	0.112
Error	999 528.80	1 152	867.65	—	—	—
Total	4 584 041.00	1 440	—	—	—	—

Note: SS = sum of squares; df = degrees of freedom; MS = mean square; η^2p = partial eta-squared (effect size); $R^2 = 0.613$; adjusted $R^2 = 0.660$.

have progressively committed to metamorphosis preparation and may rely less on continuous PTH signaling, potentially reducing their vulnerability to azadirachtin's endocrine disruption. The progressive increase in LC_{50} ($R^2 = 0.986$) across instars observed in the present study is fully consistent with this mechanistic gradient.

The most practically significant finding of this study is the abrupt ontogenetic threshold between L3 and L4, evidenced by a 271.3% increase in mean LT_{50} at this single instar transition. Previous work on *S. frugiperda* has noted reduced susceptibility of late instars to both organophosphate and pyrethroid insecticides (Carvalho et al. 2013), attributed to increased body mass, cuticular thickening, and upregulation of detoxification enzymes including cytochrome P450s (Yu 2015). The present data suggests that similar physiological transitions are also operative for botanical IGRs, and that the L3-L4 boundary represents a developmental 'tipping point' with disproportionate toxicological significance.

The three-way ANOVA result that larval instar (partial $\eta^2 = 0.399$) accounts for more variance in mortality than either concentration (partial $\eta^2 = 0.049$) or time (partial $\eta^2 = 0.355$) has important practical implications. It means that the developmental stage of the target population at the time of application is a more important determinant of outcome than the exact dose applied within the tested

range. This reinforces the case for precision timing of neem applications based on instar monitoring rather than calendar-based scheduling. In high-altitude maize systems where the slower larval development (total larval period ~ 39 days vs. ~ 20 days at 25°C) provides wider windows for intervention, this strategic flexibility is achievable.

From an IPM standpoint, these results align with the growing consensus that botanical insecticide applications targeting *S. frugiperda* are most effective when timed to early whorl infestations (Abbas et al. 2022, Anilkumar et al. 2024). However, the data also clarify that treatments applied after L3 may yield inconsistent and delayed outcomes, even at high concentrations. Growers relying on azadirachtin formulations in Andean highland systems should be advised that field applications against L4-L6 populations will require substantially longer exposure times (100-400 h) to achieve 50% mortality, a period that in field conditions may be insufficient to prevent plant damage. Complementary tactics such as *Bacillus thuringiensis* Berliner applications or the conservation of larval parasitoids may be more appropriate for managing late-instar populations in these environments (Guedes et al. 2017).

A limitation of this study is that bioassays were conducted under controlled laboratory conditions; field persistence and efficacy of azadirachtin at high altitude,

where UV radiation degradation is accelerated by thinner atmospheric filtering, requires direct evaluation. Additionally, the formulation tested contained a complex mixture of neem limonoids beyond azadirachtin A, whose individual contributions to the observed toxicity patterns are not resolved by this study. Future work should compare purified azadirachtin with full-spectrum neem formulations under high-altitude conditions to disentangle these contributions.

Conclusions

Azadirachtin retains biologically relevant insecticidal activity against *S. frugiperda* under high-mountain laboratory conditions (2 586 m a.s.l., 17°C), but its efficacy is fundamentally determined by larval ontogeny. A critical physiological threshold occurs between the third and fourth larval instars, marked by a 271.3% abrupt increase in median lethal time and a disproportionate acceleration in the LC_{50} gradient. Larval instar accounts for a larger proportion of variance in mortality (partial $\eta^2 = 0.399$) than either concentration or exposure time under the conditions tested. These findings provide the first comprehensive toxicological baseline for azadirachtin against *S. frugiperda* in Andean highland environments and define an actionable IPM decision rule: neem-based bioinsecticide applications should be targeted to L1-L3 instars to maximize efficacy, minimize input costs, and reduce the risk of control failure in high-altitude maize production systems.

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