Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Ontogenetic thresholds in larval development, azadirachtin susceptibility, and survival dynamics of *Spodoptera frugiperda* under high-mountain Andean conditions: an integrated framework for altitude-adapted IPM

Humberto Giraldo-Vanegas

humgiva64@unipamplona.edu.co

Universidad de Pamplona https://orcid.org/0000-0002-0801-2714

Sanly Natalia Núñez-García

Universidad de Pamplona https://orcid.org/0009-0000-5886-9700

Selena Marily Velásquez-López

Universidad de Pamplona https://orcid.org/0009-0005-1731-7316

Mayda Yarely Reatiga-Pulido

Universidad de Pamplona https://orcid.org/0009-0007-8048-0727

Zilenyi Vanessa Cañas-Meaury

Universidad de Pamplona https://orcid.org/0009-0008-9327-5041

Gabriel Giraldo-Herrera

Universidad de Pamplona https://orcid.org/0009-0005-1192-0069

Research Article

Keywords: Spodoptera frugiperda, azadirachtin, high-altitude agroecosystem, larval ontogeny, supernumerary instar, survival analysis, integrated pest management

Posted Date: April 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9534590/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors declare no competing interests.

Abstract

Background: *Spodoptera frugiperda* (J.E. Smith, 1797) is a globally invasive pest whose developmental biology and responses to botanical insecticides have been characterized almost exclusively under lowland tropical conditions, leaving a critical knowledge gap for high-mountain Andean agroecosystems. This study provides the first integrated characterization of larval development, azadirachtin dose-response, lethal time dynamics, and post-exposure survival of *S. frugiperda* under high-altitude laboratory conditions (2,586 m a.s.l.; 17 ± 1°C; 65 ± 10% RH).

Methods: Two independent larval cohorts were established from the same laboratory population. Cohort 1 (n = 189) characterized larval instar number, head capsule width (HCW), and developmental duration. Cohort 2 (n = 1,488; 248 per instar) was subjected to an azadirachtin bioassay (leaf-dip; 275–1,667 µL/L of 1.2% EC formulation). Phase 1 (0–96 h) yielded dose-response (Probit; LC_{50}) and lethal time (LT_{50}) estimates. Phase 2 monitored post-exposure survival until adult emergence, generating 1,009 mortality events for Kaplan–Meier (KM) and Cox proportional hazards (CPH) analyses.

Results: Under high-mountain conditions, 63% of Cohort 1 individuals completed development through a supernumerary seventh instar (total larval duration: 41.05 days). In Cohort 2, LC_{50} increased progressively from L1 (579.7 µL/L) to L6 (921.4 µL/L) (R^2 = 0.986). Mean LT_{50} increased 2.7-fold between L3 (37.9 h) and L4 (145.9 h), the largest inter-instar discontinuity. Larval instar was the dominant mortality determinant (partial η^2 = 0.399). KM confirmed cumulative mortality of 87.9–89.9% in L1–L3 versus 42.7–51.6% in L4–L6 (χ^2 = 119.35, $p < 0.001$). The CPH model showed each successive instar reduced instantaneous hazard by 15.4% (HR = 0.846).

Conclusions: High-mountain thermal conditions substantially modify larval development of *S. frugiperda*, with 63% of individuals expressing a supernumerary seventh instar. The L3–L4 ontogenetic threshold — independently supported by morphometric, dose-response, and survival analyses across two cohorts — defines a precise biological control window: azadirachtin applications in Andean high-altitude IPM programs should target L1–L3 instars to maximize cost-effectiveness.

1. Introduction

Spodoptera frugiperda (J.E. Smith, 1797) (Lepidoptera: Noctuidae) is one of the most economically damaging lepidopteran pests of cultivated crops worldwide. Native to the tropical and subtropical Americas, the species has expanded its range dramatically, establishing invasive populations across sub-Saharan Africa, South and Southeast Asia, and Australia (Tay et al. 2023; Kenis et al. 2023). In its native range, *S. frugiperda* remains a primary constraint on maize (*Zea mays* L.) production, with annual yield losses of 8.3–20.6 million metric tonnes across Africa alone when left unmanaged (Baudron et al. 2019), and comparable economic impacts documented across Latin America and the Caribbean (Stokstad 2017). Its high reproductive rate, polyphagous 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).

Although *S. frugiperda* exhibits broad climatic and altitudinal plasticity, the vast majority of studies characterizing its biology, phenology, and pest management have been conducted under lowland tropical conditions, typically between 20 and 30°C and at elevations below 1,000 m a.s.l. (Sparks 1979; Ashley et al. 1989; Nagoshi et al. 2017). This generates a critical knowledge gap for high-mountain Andean agroecosystems, where *S. frugiperda* infests maize and other crops at elevations exceeding 2,500 m a.s.l. under characteristically cool thermal regimes (15–19°C) (Yan et al. 2022). Under such conditions, both the developmental biology of the pest and the efficacy of control interventions calibrated at lowland temperatures may deviate substantially from established parameters, undermining the applicability of existing IPM recommendations.

Larval instar determination is foundational to any IPM program: instar identity conditions susceptibility to insecticides, biological control agents, and host plant resistance mechanisms, and it is the primary variable used to time control interventions in the field (Pashley et al. 1985; Nagoshi et al. 2017). In *S. frugiperda*, six larval instars are classically described under lowland conditions (Luginbill 1928; Ramírez-García et al. 2019), with head capsule widths (HCW) following Dyar's rule (Dyar 1890) and developmental duration closely coupled to temperature. However, thermal plasticity in lepidopteran larvae can induce supernumerary instars under suboptimal temperatures (Esperk et al. 2007), with direct consequences for phenological modeling and management timing. Whether *S. frugiperda* expresses such plasticity under high-mountain Andean thermal conditions has not been previously investigated.

Among botanical insecticides, azadirachtin — the principal bioactive limonoid of *Azadirachta indica* A. Juss. — has emerged as a highly relevant tool for *S. frugiperda* management in IPM programs, particularly where synthetic insecticide resistance is documented (Isman 2006; Abbas et al. 2022). Its mode of action — simultaneously antifeedant, insect growth regulatory, and endocrine disruptive — operates by interfering with the neuroendocrine cascade regulating ecdysone biosynthesis (Mordue & Nisbet 2000; Schmutterer 1990). Recent mechanistic work demonstrated that azadirachtin specifically inhibits the nuclear receptor HR3 in the prothoracic gland, blocking larval ecdysis in *S. frugiperda* (Fan et al. 2023). These effects are strongly dependent on larval instar, making azadirachtin a particularly informative model compound for investigating ontogenetic susceptibility thresholds. Nevertheless, virtually all dose-response and lethal time data for azadirachtin against *S. frugiperda* have been generated under warm lowland conditions, and its efficacy under the cooler temperatures of Andean high-altitude environments remains unquantified (Kabaru & Mwangi 2000).

The present study addresses this gap through a sequential, two-cohort experimental design. First (Cohort 1), larval development of *S. frugiperda* was characterized under high-mountain laboratory conditions to determine instar number, HCW progression, and developmental duration, with particular attention to the expression of supernumerary instars (Esperk et al. 2007). Second (Cohort 2), a continuous bioassay evaluated azadirachtin toxicity across all larval instars through Probit dose-response analysis (Finney 1971), lethal time estimation (LT_{50}), Kaplan–Meier survival analysis (Kaplan & Meier 1958), and Cox proportional hazards modelling (Cox 1972). By integrating morphometric, toxicological, and survival data from two independent cohorts of the same high-mountain population,

this study provides the first evidence-based ontogenetic framework for timing azadirachtin applications under Andean high-altitude IPM conditions.

2. Materials and Methods

2.1 Study site and environmental conditions

All experimental work was conducted at the Agronomic Engineering Laboratory, Faculty of Agricultural Sciences, Universidad de Pamplona, Pamplona, Norte de Santander, Colombia (7°22'N, 72°39'W; 2,586 m a.s.l.). Laboratory conditions were maintained at 17 ± 1°C, 65 ± 10% relative humidity (RH), and a 12:12 h light:dark photoperiod throughout all experiments. These conditions replicate the thermal regime characteristic of high-mountain Andean maize agroecosystems in the study region and represent the lowest mean temperature range reported for *S. frugiperda* bioassay studies to date (Yan et al. 2022).

2.2 Insect colony and larval cohort rearing

Two independent larval cohorts were established from a colony of *Spodoptera frugiperda* maintained at the Agronomic Engineering Laboratory of the Universidad de Pamplona (2,586 m a.s.l.), originally founded from field-collected individuals from maize crops in the municipality of Pamplona, Norte de Santander, Colombia. The colony had been maintained under the conditions described in Section 2.1 for at least three generations prior to the experiments. Larvae were reared individually in 100-mL plastic containers on a semi-artificial maize-based diet renewed every 48 h. Individual rearing was used throughout to avoid cannibalism and crowding effects (Yu 2015).

Cohort 1 (n = 189) was established to characterize larval development (Section 2.3). **Cohort 2** (n = 1,488) was established independently and used exclusively for azadirachtin bioassays (Sections 2.4–2.6). Both cohorts originated from the same laboratory population to ensure comparable genetic background, while remaining biologically independent experimental units established at different times.

2.3 Larval instar determination — Cohort 1

Larval instar number and developmental duration were determined for 189 individuals reared individually under the conditions described in Section 2.1. Head capsule width (HCW) was measured at each larval ecdysis using a calibrated ocular micrometer (10× objective) mounted on a stereomicroscope (Leica EZ4; Leica Microsystems, Wetzlar, Germany), recording the widest transverse diameter to the nearest 0.01 mm. Ecdysis events and HCW were recorded daily. Dyar's rule (Dyar 1890) was applied to evaluate the geometric progression of HCW across instars: *Growth ratio = HCW(n+1) / HCW(n)*. Individuals were classified as six-instar (canonical pathway) or seven-instar (supernumerary pathway) based on the total number of recorded ecdysis events and HCW discontinuities.

2.4 Azadirachtin source, concentrations, and bioassay design — Cohort 2

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. Seven treatments, including an absolute distilled-water control, were evaluated: 0, 275, 550, 833, 1,108, 1,383, and 1,667 µL/L of commercial product (Table 2). Bioassays were conducted using the leaf-dip method (Finney 1971): fresh maize leaf sections (5 cm²) were immersed for 30 s in each test concentration, air-dried for 60 min, and offered to individual larvae confined in 9-cm-diameter plastic Petri dishes. Twenty-five larvae per concentration per instar were tested, yielding 1,050 total experimental units across six instars.

2.5 Phase 1: Dose-response and lethal time analysis (0–96 h)

Larval mortality was recorded at 12, 24, 48, 72, and 96 h post-treatment. Abbott's correction formula was applied to adjust for control mortality (Abbott 1925, cited in Finney 1971). Dose-response data at 96 h were analyzed by Probit regression (Finney 1971) using SPSS v.26.0 (IBM Corp., Armonk, NY, USA) to estimate LC_{50} and LC_{95} with 95% confidence intervals (CI) for each larval instar (Table 4). Lethal time (LT_{50}) values were estimated by fitting Probit regression equations to time-mortality data at each concentration and instar (Tables 5–10). A three-way ANOVA was conducted to evaluate the effects of larval instar (6 levels), concentration (6 treatment levels), and exposure time (8 recording intervals) on mortality using Type III sums of squares and partial η^2 as an effect size measure (Table 11). Post-hoc comparisons used Tukey's HSD test ($\alpha = 0.05$).

2.6 Phase 2: Survival analysis (96 h to adult emergence or death)

Following the 96 h observation period, all surviving individuals were maintained individually under the same conditions and monitored daily until adult emergence or death, yielding 1,488 time-to-event records (1,009 mortality events; 479 right-censored observations). Survival functions for each larval instar were estimated using the Kaplan–Meier (KM) estimator (Kaplan & Meier 1958), with pairwise log-rank tests and Bonferroni correction. The Cox proportional hazards (CPH) model (Cox 1972) was fitted with larval instar and azadirachtin concentration as continuous covariates. The proportional hazards assumption was verified by Schoenfeld residuals analysis. All survival analyses were performed using SPSS v.26.0.

3. Results

3.1 Larval development under high-mountain conditions — Cohort 1

Of the 189 larvae evaluated under high-mountain laboratory conditions (17 ± 1°C; 65 ± 10% RH), two distinct developmental pathways were identified: 70 individuals (37%) completed development through the canonical six-instar pathway, while 119 individuals (63%) expressed a supernumerary seventh instar (Table 1). The seven-instar pathway was thus the numerically dominant developmental pattern under the thermal conditions tested.

In the **six-instar pathway**, mean total larval duration was 39.17 days, with individual instar durations ranging from 3.67 days (L2) to 13.63 ± 1.75 days (L6) (Table 1). Of 70 individuals, 58 (82.9%) successfully pupated, with a mean pupal duration of 21.05 ± 1.66 days.

In the **seven-instar pathway**, mean total larval duration was 41.05 days. Individual instar durations ranged from 3.52 days (L2) to 11.59 ± 0.77 days (L7). The redistribution of developmental time across seven instars resulted in a shorter terminal instar (L7: 11.59 days) compared to L6 in the canonical pathway (13.63 days), suggesting that the supernumerary instar partitions rather than simply extends the final growth phase (Esperk et al. 2007). Of 119 individuals, 96 (80.7%) successfully pupated, with a mean pupal duration of 21.91 ± 1.48 days.

Table 1. Developmental duration (days ± SD) for each larval instar and pupal stage of *Spodoptera frugiperda* reared under high-mountain laboratory conditions (17 ± 1°C, 65 ± 10% RH, 12:12 h L:D). Data are shown for both the six-instar (canonical) and seven-instar (supernumerary) developmental pathways (Cohort 1). Selected ages for Cohort 2 bioassays are given in the final column.

Instar	n (6-instar)	Duration 6-instar (days ± SD)	Cumul. age 6-instar (days)	n (7-instar)	Duration 7-instar (days ± SD)	Cumul. age 7-instar (days)	Selected age for bioassay (days)
L1	70	5.19 ± 0.39	5.19	119	5.30 ± 0.46	5.30	3 ± 0.5
L2	70	3.67 ± 0.53	8.86	119	3.52 ± 0.53	8.82	7 ± 0.5
L3	70	4.36 ± 0.54	13.22	119	4.09 ± 0.34	12.91	11 ± 0.5
L4	70	5.93 ± 0.46	19.15	119	5.41 ± 0.56	18.32	17 ± 0.5
L5	70	6.39 ± 1.22	25.54	119	4.97 ± 0.75	23.29	22 ± 0.5
L6	70	13.63 ± 1.75	39.17	119	6.17 ± 0.90	29.46	35 ± 1.0
L7	—	—	—	119	11.59 ± 0.77	41.05	—
Total larval	70	39.17	—	119	41.05	—	—
Pupa	58	21.05 ± 1.66	—	96	21.91 ± 1.48	—	—

Note: Cumulative age calculated as the sum of mean instar durations. Selected ages for bioassay were defined to minimize overlap between consecutive instars.

3.2 Control mortality and general dose-mortality response — Cohort 2

Control-corrected mortality remained consistently low across all six instars (mean 0.66%; range 0.00–4.00%), confirming bioassay validity (Table 3). The integrated Probit model combining all instars yielded $R^2 = 0.89$, with a general LC_{50} of 740.4 µL/L of commercial product (equivalent to 8.88 mg/L technical azadirachtin). This value falls within the range reported for similar commercial formulations under lowland tropical conditions (600–1,200 µL/L) (Silva et al. 2020; Tulashie et al. 2021), suggesting that the cooler high-mountain thermal regime does not substantially compromise azadirachtin efficacy against early instars.

Table 2. Azadirachtin concentrations evaluated in the toxicological bioassay (Neem-X 1.2% EC formulation; 12 g azadirachtin/L). n = 25 larvae per concentration per instar; total = 1,050 experimental units across six instars (Cohort 2).

Treatment	Commercial product (mL/L)	Applied azadirachtin (µL/L)	Technical azadirachtin (mg/L)
T1 (Control)	0.00	0	0.00
T2	0.275	275	3.30
T3	0.550	550	6.60
T4	0.833	833	10.00
T5	1.108	1,108	13.30
T6	1.383	1,383	16.60
T7	1.667	1,667	20.00

Table 3. Control mortality (%) and total mortality (%) at 96 h by larval instar of *Spodoptera frugiperda* exposed to six concentrations of azadirachtin. n = 25 larvae per instar per treatment; Abbott's correction applied. Values followed by different letters within each column are significantly different (Tukey's HSD, $p \leq 0.05$).

Concentration (µL/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

3.3 Instar-specific LC_{50} and the ontogenetic susceptibility 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 4). First-instar larvae were the most susceptible (LC_{50} = 579.7 µL/L; 95% CI: 512.3–655.2), whereas sixth-instar larvae required a 59.0% higher concentration (LC_{50} = 921.4 µL/L; 95% CI: 835.1–1,015.7) to achieve 50% mortality at 96 h. Despite this continuous increase, the critical acceleration occurred at the L3–L4 transition: the proportional LC_{50} increase from L3 to L4 (13.2%) was equivalent to the cumulative increase across L1–L3 combined (13.2%), indicating an abrupt acceleration of the resistance gradient at this developmental point.

Table 4. Probit regression equations, coefficients of determination (R^2), median lethal concentration (LC_{50}), and 95% confidence intervals (CI) of azadirachtin (Neem-X 1.2% EC) for each larval instar of *Spodoptera frugiperda* at 96 h of exposure (Cohort 2, Phase 1). LC_{50} values in µL/L of commercial product.

Instar	Probit equation	R^2	$\log_{10}(LC_{50})$	LC_{50} (µL/L)	95% CI (µL/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
Overall	Y = 3.18X − 4.17	0.89	2.87	740.4	689.2–795.6

Note: Y = Probit (mortality); X = $\log_{10}$ (concentration in µL/L). Linear regression of LC_{50} on instar: R^2 = 0.986, $p < 0.001$; LC_{50} = 472.8 + 74.3 × instar.

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

LT_{50} analyses revealed a markedly more pronounced ontogenetic discontinuity than LC_{50} data (Tables 5–10). 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 single largest inter-instar jump occurred at the L3–L4 transition: 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. Even at the highest concentration tested (1,667 µL/L), L5 larvae required a median time of 95.2 h to reach 50% mortality.

Table 5. Median lethal time (LT_{50}), Probit regression equations, and R^2 for first-instar (L1) larvae of *Spodoptera frugiperda* exposed to azadirachtin (Cohort 2, Phase 1).

Concentration (µL/L)	Probit 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
Mean	—	—	52.37	—

Table 6. LT_{50}, Probit regression equations, and R^2 for second-instar (L2) larvae of *Spodoptera frugiperda* exposed to azadirachtin (Cohort 2, Phase 1).

Concentration (µL/L)	Probit 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
Mean	—	—	39.55	—

Table 7. LT_{50}, Probit regression equations, and R^2 for third-instar (L3) larvae of *Spodoptera frugiperda* exposed to azadirachtin. Note: L3 mean LT_{50} (37.93 h) is 2.7-fold lower than L4 (145.87 h), marking the critical L3–L4 physiological threshold.

Concentration (µL/L)	Probit 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
Mean	—	—	37.93	—

Table 8. LT_{50}, Probit regression equations, and R^2 for fourth-instar (L4) larvae of *Spodoptera frugiperda* exposed to azadirachtin.

Concentration (µL/L)	Probit 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
Mean	—	—	145.96	—

Table 9. LT_{50}, Probit regression equations, and R^2 for fifth-instar (L5) larvae of *Spodoptera frugiperda* exposed to azadirachtin.

Concentration (μL/L)	Probit 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
Mean	—	—	196.69	—

Table 10. LT_{50}, Probit regression equations, and R^2 for sixth-instar (L6) larvae of *Spodoptera frugiperda* exposed to azadirachtin.

Concentration (μL/L)	Probit 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
Mean	—	—	135.77	—

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

The three-way ANOVA confirmed that all three main factors significantly influenced larval mortality (Table 11). Larval instar was the dominant 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 $\eta^2 = 0.049$, $F_{5,1152} = 11.79$, $p < 0.001$), together accounting for 80.3% of total explainable variance. Two-way interactions between instar × concentration (partial $\eta^2 = 0.063$, $p < 0.001$) and instar × time (partial $\eta^2 = 0.087$, $p < 0.001$) were both significant. Concentration × time interaction was non-significant ($p = 0.249$), and the three-way interaction was also non-significant ($p = 0.953$), confirming that

developmental stage is the primary modulator of azadirachtin efficacy regardless of the concentration–time combination applied (Table 11).

Table 11. Three-way ANOVA of the effects of larval instar, azadirachtin concentration, and exposure time on mortality of *Spodoptera frugiperda* (Cohort 2, Phase 1). Type III sums of squares; α = 0.05.

Source of variation	Type III SS	df	MS	F	p-value	η²p
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 × Concentration	67,286.27	25	2,691.45	3.10	<0.001	0.063
Instar × Time	94,741.16	35	2,706.89	3.12	<0.001	0.087
Concentration × Time	35,029.66	35	1,000.85	1.15	0.249	0.034
Instar × Conc. × 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; η²p = partial eta-squared; R^2 = 0.613.

3.6 Survival analysis: Kaplan–Meier curves and Cox proportional hazards model

Kaplan–Meier survival curves differed significantly among all six larval instars (combined log-rank trend test: $\chi^2 = 119.35$, df = 1, $p < 0.001$), with a monotonic increase in survival probability with advancing instar. Cumulative mortality within the full observation period reached 87.9–89.9% in L1–L3 but declined sharply to 42.7–51.6% in L4–L6, with more than half of late-instar individuals surviving to adult emergence or the end of the observation period. Pairwise log-rank comparisons with Bonferroni correction identified a significant survival discontinuity specifically at the L3–L4 boundary ($p < 0.001$), while differences within the L1–L3 and L4–L6 groups were comparatively minor. This result independently confirms and extends the L3–L4 ontogenetic threshold identified by dose-response and LT_{50} analyses.

The CPH model incorporating larval instar and azadirachtin concentration as continuous covariates was highly significant ($\chi^2 = 95.77$, df = 2, $p < 0.001$), and the proportional hazards assumption was satisfied for both covariates (Schoenfeld residuals test: $p > 0.05$). Each successive larval instar reduced the

instantaneous mortality hazard by 15.4% (HR = 0.846; 95% CI: 0.816–0.878), independently of concentration. Each 100 µL/L concentration increment increased the instantaneous hazard by 2.6% (HR = 1.026; 95% CI: 1.013–1.039).

3.7 Convergence of the L3–L4 ontogenetic threshold across three analytical frameworks

The L3–L4 susceptibility threshold was independently identified by three analytical approaches across two experimental cohorts: (1) **Morphometric analysis (Cohort 1)**: the L3–L4 transition coincides with the developmental boundary at which the dominant pathway shifts from six- to seven-instar development (Table 1; Esperk et al. 2007); (2) **Probit dose-response analysis (Cohort 2)**: an abrupt 13.2% acceleration in the LC_{50} gradient at L3→L4 — equivalent to the cumulative increase across all of L1–L3 combined — signals a qualitative change in susceptibility (Table 4); (3) **Survival analysis (Cohort 2)**: a statistically significant and biologically abrupt change in both cumulative mortality (Kaplan–Meier) and instantaneous hazard (CPH model) occurs at the same developmental boundary. The convergence of three independent lines of evidence across two separate cohorts from the same field population constitutes robust multimethodological support for the L3–L4 transition as a genuine physiological threshold with direct implications for IPM timing decisions.

4. 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 µL/L (Table 4) is consistent with values reported under Brazilian lowland conditions (600–1,200 µL/L for similar commercial formulations) (Silva et al. 2020; 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 classical finding that neem extracts exhibit markedly reduced toxicity against Orthoptera at temperatures below 22°C (Kabaru & Mwangi 2000), and may reflect the fact that azadirachtin's primary mode of action — disruption of hormonal molting cascades rather than neurotoxicity — is less temperature-dependent (Mordue & Nisbet 2000; Schmutterer 1990).

The molecular basis for the high susceptibility of 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 actively depend on rapid molt cycles and whose prothoracic gland activity is at its peak (Mordue & Nisbet 2000). The progressive and near-linear increase in LC_{50} across instars (R^2 = 0.986; Table 4) is fully consistent with this mechanistic gradient.

The most practically significant finding is the abrupt ontogenetic threshold between L3 and L4, evidenced by a 271.3% increase in mean LT_{50} at this single instar transition (Tables 7 and 8). Previous work on *S. frugiperda* has noted reduced susceptibility of late instars to organophosphates and pyrethroids (Carvalho et al. 2013), attributed to increased body mass, cuticular thickening, and upregulation of cytochrome P450 detoxification enzymes (Yu 2015). The present data indicate that similar physiological transitions also operate for botanical IGRs. Importantly, the altitude-driven expression of a supernumerary seventh instar in 63% of Cohort 1 individuals (Table 1) extends the period spent as early instars under high-mountain conditions, potentially widening the practical window for effective azadirachtin application relative to lowland populations (Esperk et al. 2007; Yan et al. 2022).

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 $\eta^2 = 0.355$) (Table 11) has critical practical implications: the developmental stage of the target population at the time of application is a more important outcome determinant 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 (Anilkumar et al. 2024). In high-altitude maize systems, the slower larval development documented in this study (~39–41 days total vs. ~20 days at 25°C) (Yan et al. 2022; Zanzana et al. 2024) provides wider windows for intervention, making instar-based timing more operationally achievable than in lowland systems.

The Kaplan–Meier and Cox proportional hazards analyses provide an additional and methodologically independent confirmation of the L3–L4 threshold. The abrupt shift in KM cumulative mortality from 87.9–89.9% in L1–L3 to 42.7–51.6% in L4–L6, combined with the CPH hazard ratio of 0.846 per successive instar, quantifies for the first time the magnitude of the ontogenetic protective effect in terms of instantaneous survival probability. The non-significant concentration × time interaction ($p = 0.249$; Table 11) further indicates that increasing dose within the tested range does not substantially accelerate mortality beyond the instar-determined baseline — a result that argues against compensating for advanced larval stage with higher doses as a management strategy (Guedes et al. 2017).

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; Kenis et al. 2023; Anilkumar et al. 2024). The data specifically clarify that applications after L3 may yield inconsistent and delayed outcomes even at high concentrations, with LT_{50} values for L4–L6 reaching 92–410 h (Tables 8–10) — a period under field conditions may be insufficient to prevent yield-affecting plant damage. Complementary tactics, such as *Bacillus thuringiensis* Berliner applications or conservation and augmentative release of larval parasitoids, may be more appropriate for managing late-instar populations in these environments (Guedes et al. 2017). The integration of the instar-specific toxicological profile documented here with phenological models calibrated to high-altitude Andean degree-day accumulation should be a priority for future applied entomology research in these systems (Zanzana et al. 2024).

5. Conclusions

High-mountain thermal conditions substantially modify larval development of *Spodoptera frugiperda*, with 63% of individuals expressing a supernumerary seventh instar and a total larval period of ~41 days — approximately twice the duration reported under lowland conditions (Table 1). Azadirachtin retains biologically relevant insecticidal activity under these conditions, but its efficacy is fundamentally determined by larval ontogeny. A critical physiological threshold at the L3–L4 transition — marked by a 271.3% increase in mean LT_{50}, a proportional LC_{50} acceleration equivalent to the entire L1–L3 cumulative gradient, and an abrupt discontinuity in both KM survival curves and Cox hazard ratios — defines an actionable IPM decision rule: azadirachtin applications in Andean high-altitude maize systems should target L1–L3 instars to maximize efficacy, minimize input costs, and reduce the risk of control failure. The convergence of three independent analytical lines of evidence across two separate experimental cohorts represents, to our knowledge, the most robust multimethodological characterization of an ontogenetic insecticide susceptibility threshold in this species to date.

Declarations

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Funding

This research was supported internally by the Universidad de Pamplona through the provision of laboratory spaces and equipment for the Sustainable Plant Health Research Seedbed (Semillero de Investigación en Sanidad Vegetal Sustentable). No external funding was received for this study.

Ethics approval

This study did not involve human participants or vertebrate animals. All experimental procedures involving insects were conducted in accordance with institutional guidelines for invertebrate research at the Universidad de Pamplona. No specific permits were required for the collection and laboratory maintenance of *S. frugiperda*, as this species is an agricultural pest in Colombia and is not subject to conservation protection measures.

Author contributions

Conceptualization: H.G.-V.; Methodology: H.G.-V., S.N.N.-G., S.M.V.-L.; Investigation: S.N.N.-G., S.M.V.-L. (Cohort 1), M.Y.R.-P., Z.V.C.-M. (Cohort 2); Formal analysis: H.G.-V., G.G.-H.; Data curation: H.G.-V., M.Y.R.-P., Z.V.C.-M., G.G.-H.; Writing — original draft: H.G.-V.; Writing — review & editing: all authors; Supervision:

H.G.-V.; Project administration: H.G.-V. All authors have read and agreed to the published version of the manuscript.

Data availability

The raw datasets generated and analyzed during this study are available from the corresponding author (H. Giraldo-Vanegas; humgiva64@unipamplona.edu.co) upon reasonable request. Statistical analysis scripts and survival datasets will be deposited in a public repository (Dryad Digital Repository) following journal acceptance.

Preprint disclosure

Preliminary versions of this work were deposited as preprints on Research Square prior to journal submission. These preprints represent earlier stages of the research and contain subsets of the data presented in this integrated manuscript (Giraldo-Vanegas et al. 2024a, b, c).

References

1. Abbas A, Ullah F, Hafeez M, et al (2022) Biological control of fall armyworm, Spodoptera frugiperda. Agronomy 12:2704. https://doi.org/10.3390/agronomy12112704
2. Anilkumar G, LakshmiSoujanya P, Kumar DV, et al (2024) Integrated approaches for the management of invasive fall armyworm, Spodoptera frugiperda, in maize. J Plant Dis Prot 131:793–803. https://doi.org/10.1007/s41348-023-00793-9
3. Ashley TR, Wiseman BR, Davis FM, Andrews KL (1989) The fall armyworm: a bibliography. Fla Entomol 72:152–202. https://doi.org/10.2307/3495185
4. Baudron F, Zaman-Allah MA, Chaipa I, Chari N, Chinwada P (2019) Understanding the factors influencing fall armyworm (Spodoptera frugiperda J.E. Smith) damage in African smallholder maize fields and quantifying its impact on yield. Crop Prot 120:141–150. https://doi.org/10.1016/j.cropro.2019.01.028
5. Carvalho RA, Omoto C, Field LM, Williamson MS, Bass C (2013) Investigating the molecular mechanisms of organophosphate and pyrethroid resistance in the fall armyworm Spodoptera frugiperda. Pestic Biochem Physiol 106:1–9. https://doi.org/10.1016/j.pestbp.2013.01.002
6. Cox DR (1972) Regression models and life-tables. J R Stat Soc Ser B 34:187–220
7. Dyar HG (1890) The number of molts of lepidopterous larvae. Psyche 5:420–422
8. Esperk T, Tammaru T, Nylin S (2007) Intraspecific variability in number of larval instars in insects. J Econ Entomol 100:627–645. https://doi.org/10.1603/0022-0493(2007)100[627:IVINOL]2.0.CO;2
9. Fan ST, Wu MZ, Liu C, et al (2023) Azadirachtin inhibits nuclear receptor HR3 in the prothoracic gland to block larval ecdysis in the fall armyworm, Spodoptera frugiperda. J Agric Food Chem

71:15497–15505. https://doi.org/10.1021/acs.jafc.3c05508

10. Finney DJ (1971) Probit Analysis, 3rd edn. Cambridge University Press, Cambridge
11. Giraldo-Vanegas H, Réatiga-Pulido MY, Cañas-Meaury ZV (2024a) Azadirachtin toxicity against larval instars of Spodoptera frugiperda (J.E. Smith): ontogenetic thresholds and lethal time dynamics under high-mountain laboratory conditions. Res Square [Preprint]. https://doi.org/10.21203/rs.3.rs-9131584/v1
12. Giraldo-Vanegas H, Réatiga-Pulido MY, Cañas-Meaury ZV (2024b) Ontogenetic variation in the susceptibility of Spodoptera frugiperda (Lepidoptera: Noctuidae) to azadirachtin under high-altitude conditions: an integrated dose-response approach and survival analysis. Res Square [Preprint]. https://doi.org/10.21203/rs.3.rs-9003650/v1
13. Giraldo-Vanegas H, Núñez-García SN, Velásquez-López SM (2024c) Influence of altitude on larval instar determination of Spodoptera frugiperda (Lepidoptera: Noctuidae) under high-mountain laboratory conditions. Res Square [Preprint]. https://doi.org/10.21203/rs.3.rs-8876098/v1
14. Guedes RNC, Smagghe G, Stark JD, Desneux N (2017) Pesticide-induced stress in arthropod pests for optimized integrated pest management programs. Annu Rev Entomol 62:43–62. https://doi.org/10.1146/annurev-ento-010715-023601
15. Isman MB (2006) Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol 51:45–66. https://doi.org/10.1146/annurev.ento.51.110104.151146
16. Kabaru JM, Mwangi RW (2000) Effect of post-treatment temperature on the insecticidal activity of neem, Azadirachta indica A. Juss. seed extract on Schistocerca gregaria (Forskal). Int J Trop Insect Sci 20:163–167. https://doi.org/10.1017/S1742758400020610
17. Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481
18. Kenis M, Benelli G, Biondi A, et al (2023) Invasiveness, biology, ecology, and management of the fall armyworm, Spodoptera frugiperda. Entomol Gen 43:187–241. https://doi.org/10.1127/entomologia/2022/1659
19. Luginbill P (1928) The fall army worm. USDA Technical Bulletin 34:1–91
20. Mordue (Luntz) AJ, Nisbet AJ (2000) Azadirachtin from the neem tree Azadirachta indica: its actions against insects. An Soc Entomol Bras 29:615–632. https://doi.org/10.1590/S0301-80592000000400001
21. Nagoshi RN, Meagher RL, Hay-Roe M (2017) Inferring the annual migration patterns of fall armyworm (Lepidoptera: Noctuidae) in the United States from mitochondrial haplotypes. Ecol Evol 2:1458–1467. https://doi.org/10.1002/ece3.268
22. Pashley DP, Johnson SJ, Sparks AN (1985) Genetic population structure of migratory moths: the fall armyworm (Lepidoptera: Noctuidae). Ann Entomol Soc Am 78:756–762. https://doi.org/10.1093/aesa/78.6.756

23. Ramírez-García A, et al (2019) Molecular identification of fall armyworm (Spodoptera frugiperda) (Lepidoptera: Noctuidae) strains in maize of tropical regions of Veracruz, Mexico. Southwest Entomol 44:417–424. https://doi.org/10.3958/059.044.0210
24. Schmutterer H (1990) Properties and potential of natural pesticides from the neem tree, Azadirachta indica. Annu Rev Entomol 35:271–297. https://doi.org/10.1146/annurev.en.35.010190.001415
25. Silva DM, Bueno AF, Andrade K, Stecca CS, Neves PMOJ (2020) Toxicity of botanical insecticides to Spodoptera frugiperda and implications for integrated pest management. Crop Prot 137:105262. https://doi.org/10.1016/j.cropro.2020.105262
26. Sparks AN (1979) A review of the biology of the fall armyworm. Fla Entomol 62:82–87
27. Stokstad E (2017) New crop pest takes Africa at lightning speed. Science 356:473–474. https://doi.org/10.1126/science.356.6337.473
28. Tay WT, Meagher RL Jr, Czepak C, Groot AT (2023) Spodoptera frugiperda: ecology, evolution, and management options of an invasive species. Annu Rev Entomol 68:299–317. https://doi.org/10.1146/annurev-ento-120220-102548
29. Tulashie SK, Adjei F, Abraham J, Addo E (2021) Potential of neem extracts as natural insecticide against fall armyworm (Spodoptera frugiperda). Case Stud Chem Environ Eng 4:100130. https://doi.org/10.1016/j.cscee.2021.100130
30. Yan XR, Wang ZY, Feng SQ, Zhao ZH, Li ZH (2022) Impact of temperature change on the fall armyworm, Spodoptera frugiperda, under global climate change. Insects 13:981. https://doi.org/10.3390/insects13110981
31. Yu SJ (2015) The Toxicology and Biochemistry of Insecticides. CRC Press, Boca Raton
32. Zanzana K, Dossou EA, Adda AS, Tossou JM (2024) Fall armyworm management in a changing climate: an overview of climate-responsive IPM strategies. Egypt J Biol Pest Control 34:102. https://doi.org/10.1186/s41938-024-00866-x

Figures

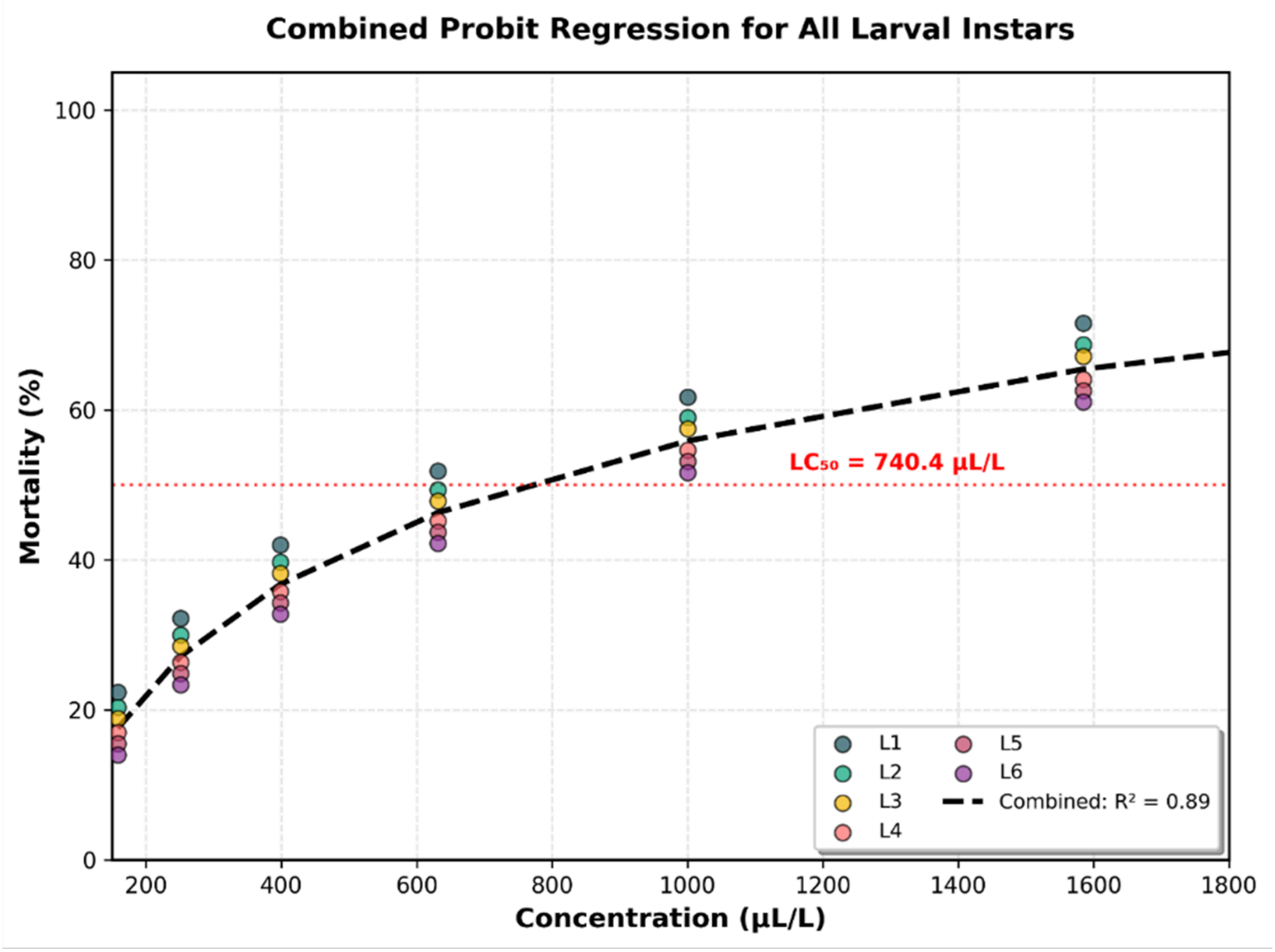


Figure 1

Combined Probit dose–response regression for all six larval instars of *Spodoptera frugiperda* exposed to azadirachtin under high-mountain laboratory conditions (2,586 m a.s.l.; 17 ± 1°C). Each point represents observed corrected mortality at 96 h for a given concentration–instar combination. The dashed regression line represents the integrated Probit model (R^2 = 0.89; general LC_{50} = 740.4 µL/L). The dotted horizontal line marks the 50% mortality threshold.

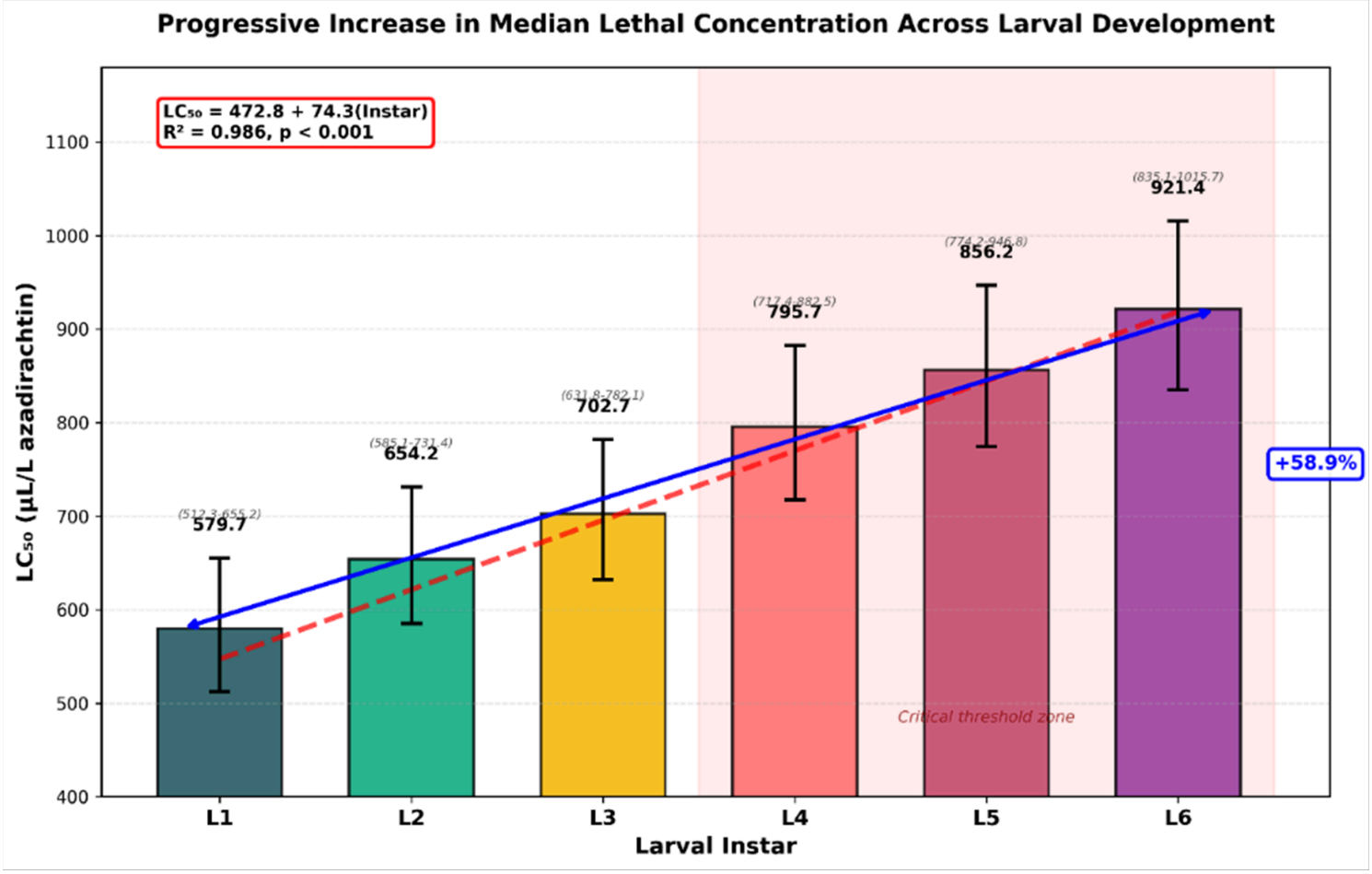


Figure 2

Median lethal concentration (LC_{50}) of azadirachtin (Neem-X 1.2% EC) across six larval instars of *S. frugiperda*at 96 h of exposure under high-mountain conditions (17 ± 1°C; 2,586 m a.s.l.). Bars represent LC_{50} values with 95% confidence intervals. The progressive increase from L1 (579.7 µL/L) to L6 (921.4 µL/L) follows a linear relationship ($R^2 = 0.986$, $p < 0.001$). The shaded area indicates the critical threshold zone at the L3–L4 transition.

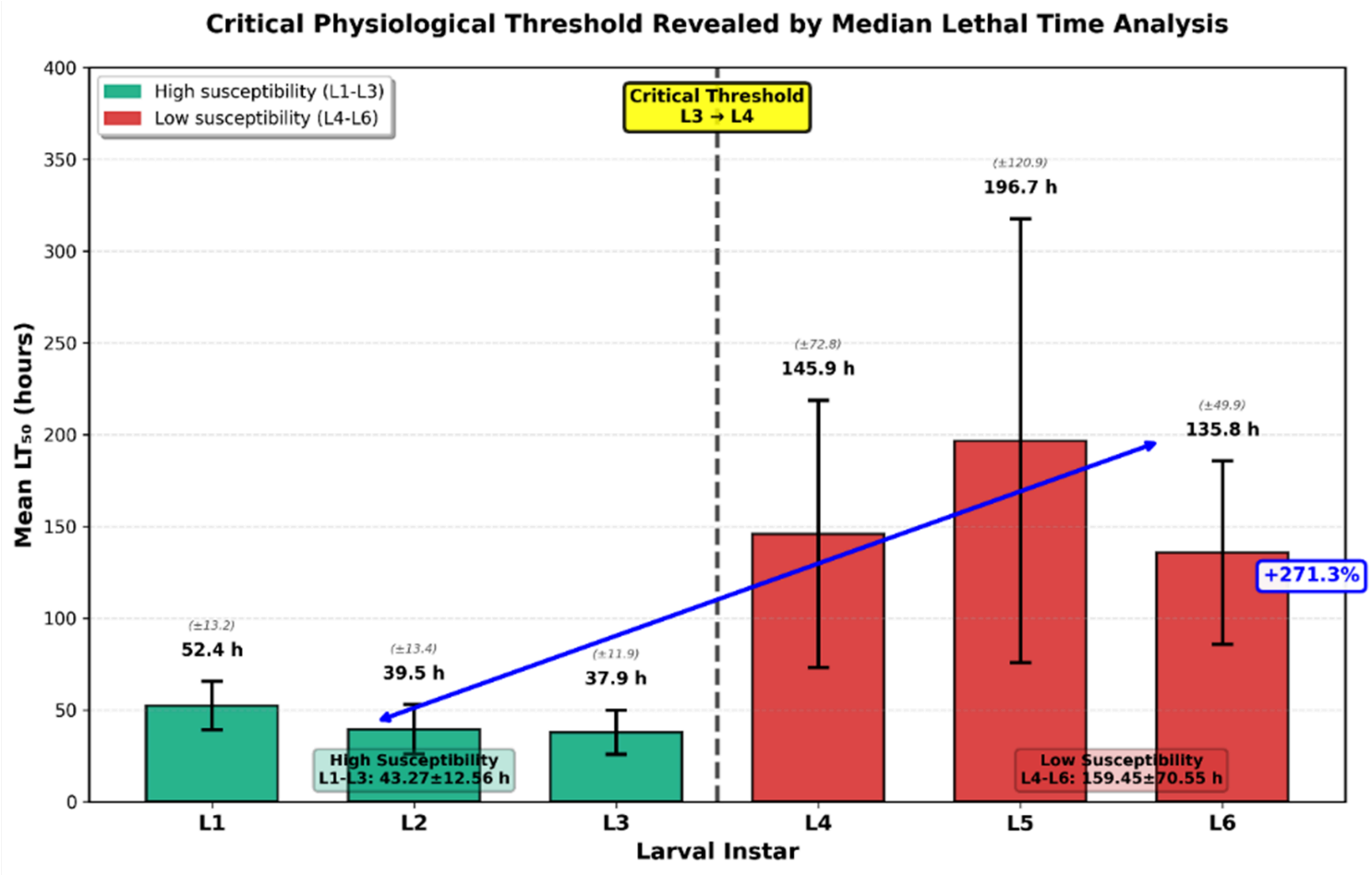


Figure 3

Mean lethal time (LT_{50}) across six larval instars of *S. frugiperda* exposed to azadirachtin under high-mountain conditions. Green bars = early instars (L1–L3; mean LT_{50} = 43.3 ± 12.6 h); red bars = late instars (L4–L6; mean LT_{50} = 159.5 ± 70.6 h). Error bars = standard deviation across concentrations (Tables 5–10). The dashed vertical line marks the L3→L4 threshold, corresponding to a 271.3% increase in mean LT_{50}.

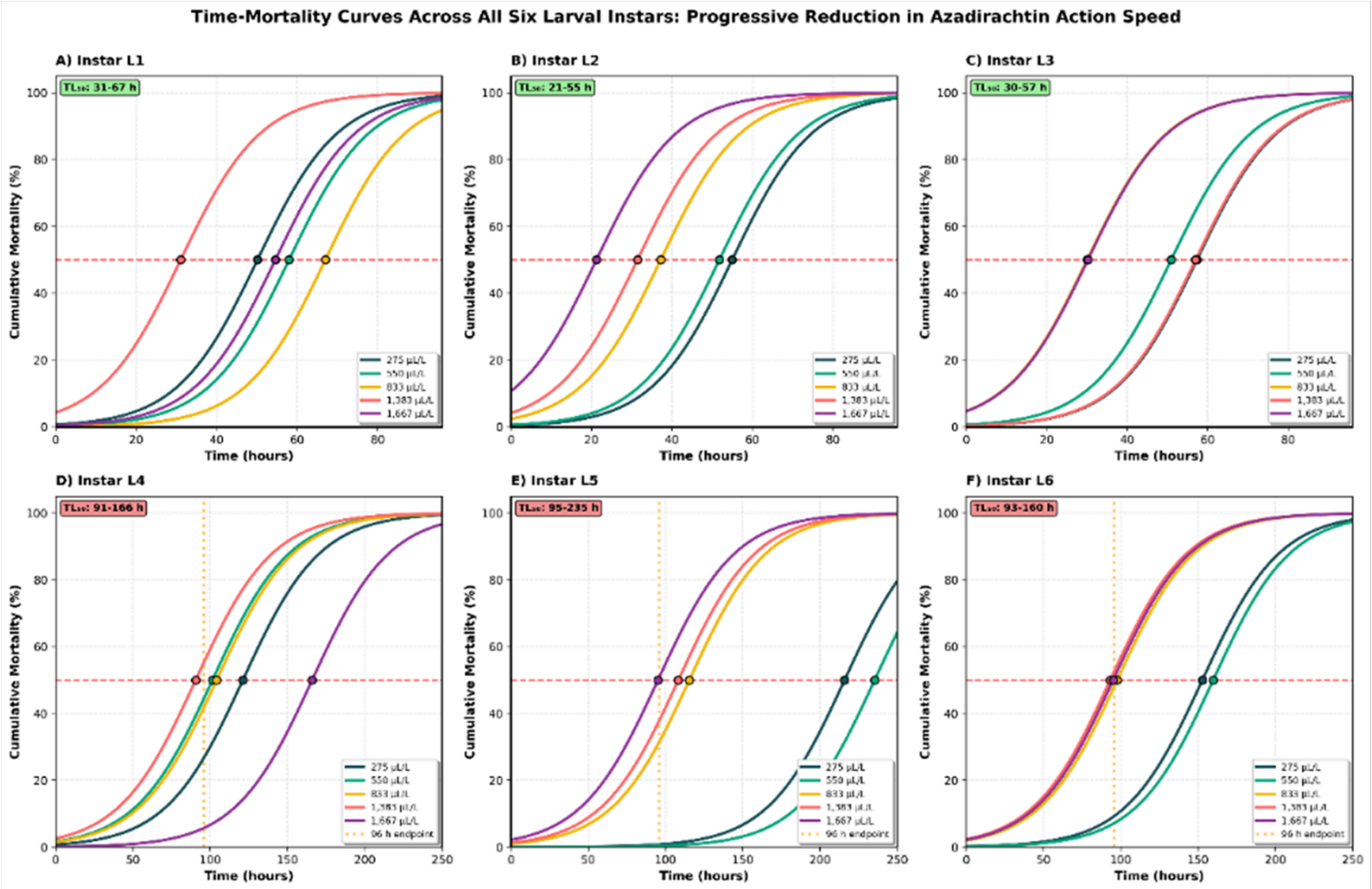


Figure 4

Time–mortality curves for six larval instars (A–F) of *S. frugiperda* exposed to azadirachtin at six concentrations (275–1,667 µL/L) under high-mountain conditions. Early instars (L1–L3) show steep sigmoidal curves reaching 100% mortality within 96 h; late instars (L4–L6) show flattened curves extending beyond 96 h.

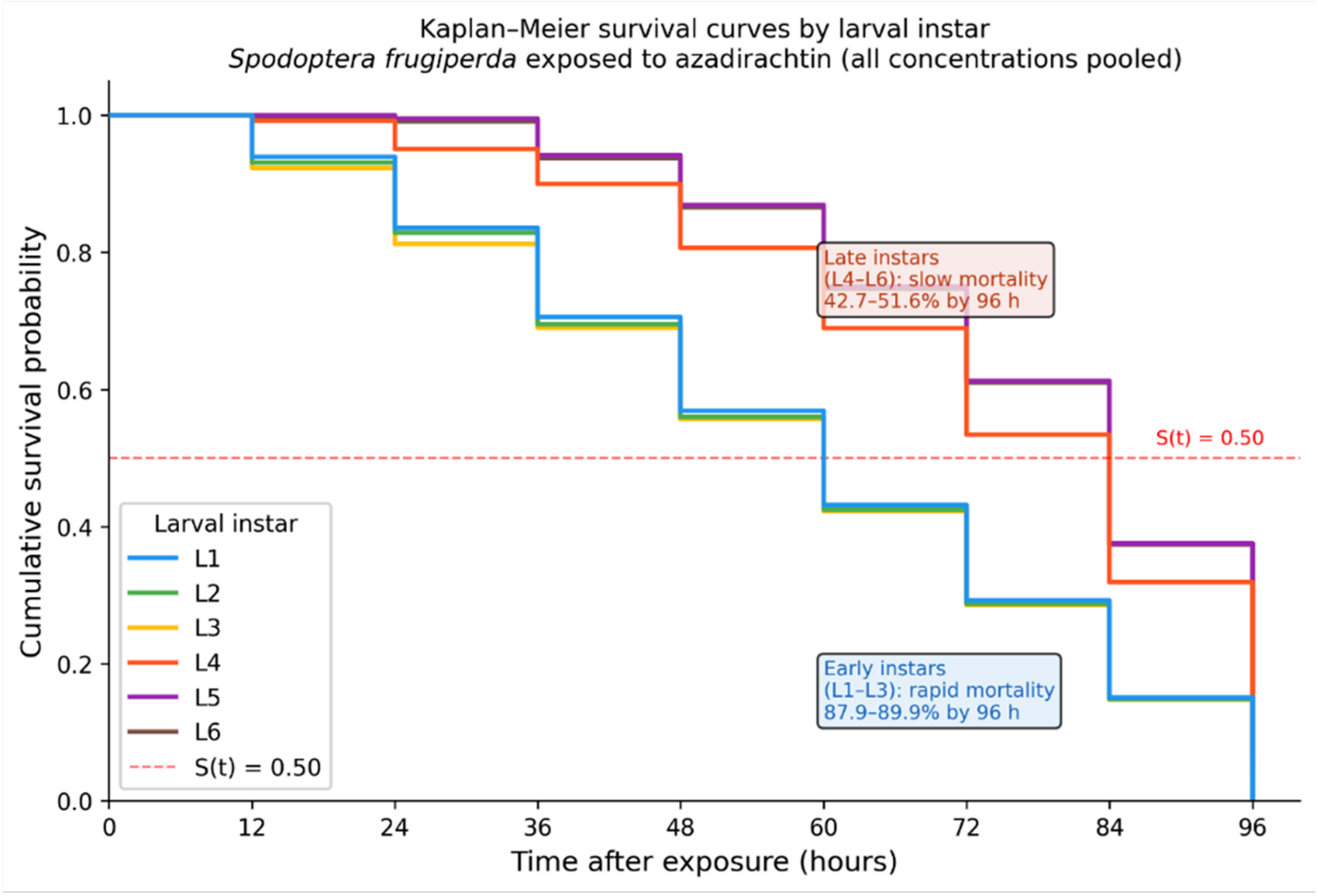


Figure 5

Kaplan–Meier survival curves for six larval instars of *S. frugiperda* exposed to azadirachtin under high-mountain laboratory conditions (Cohort 2, Phase 2; n = 248 per instar; 1,488 time-to-event records total). Each curve represents one larval instar (L1–L6). Crosses (+) indicate right-censored observations. The combined log-rank trend test was statistically significant ($\chi^2 = 119.35$, df = 1, $p < 0.001$). The dashed vertical line marks the significant survival discontinuity at the L3–L4 boundary ($p < 0.001$).

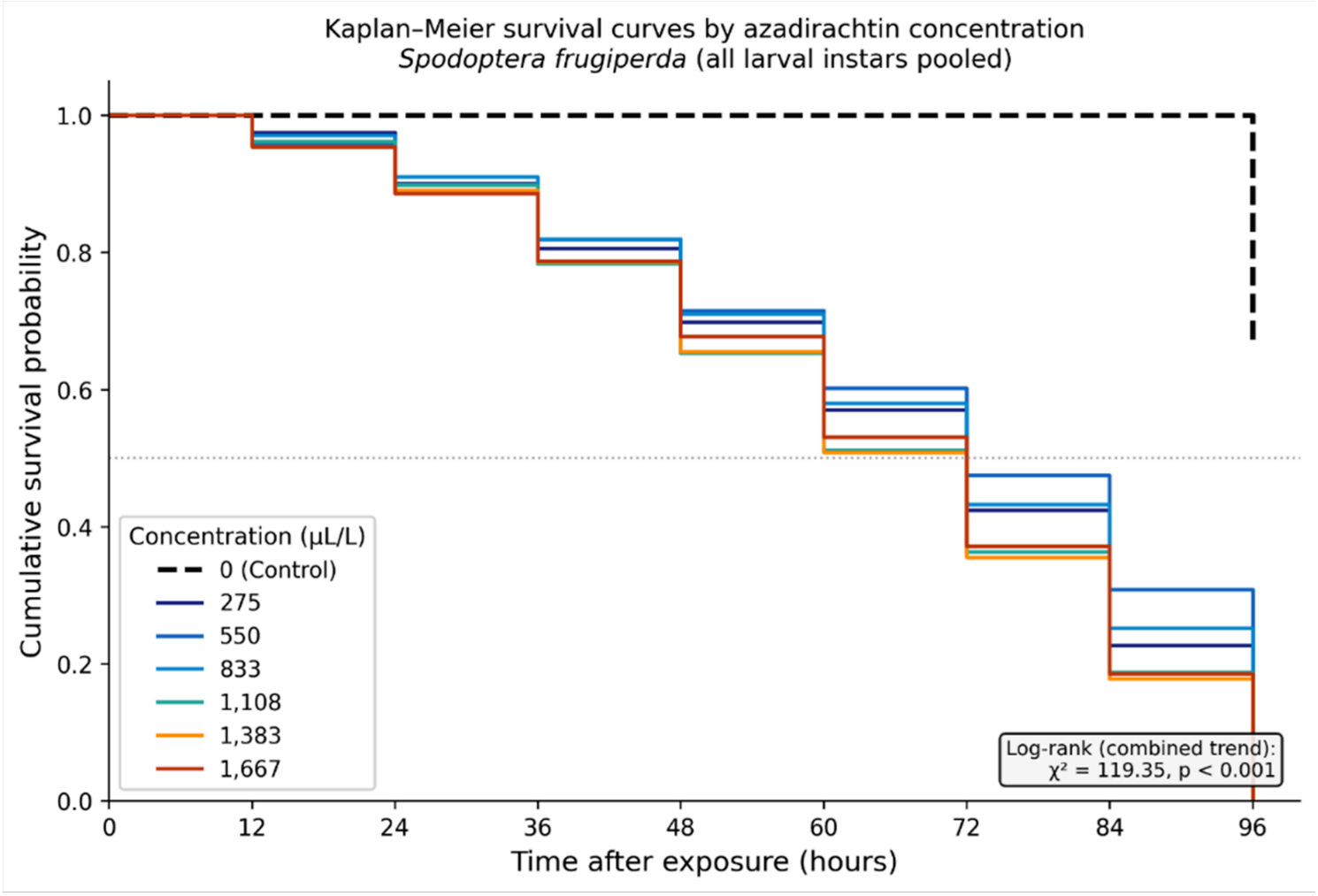


Figure 6

Kaplan–Meier survival curves stratified by azadirachtin concentration (0–1,667 µL/L of 1.2% EC formulation) for all larval instars of *S. frugiperda* pooled. The control group (0 µL/L, dashed line) maintained near-complete survival throughout 96 h. All treated concentrations produced significant reductions in survival relative to controls (log-rank $p < 0.001$).

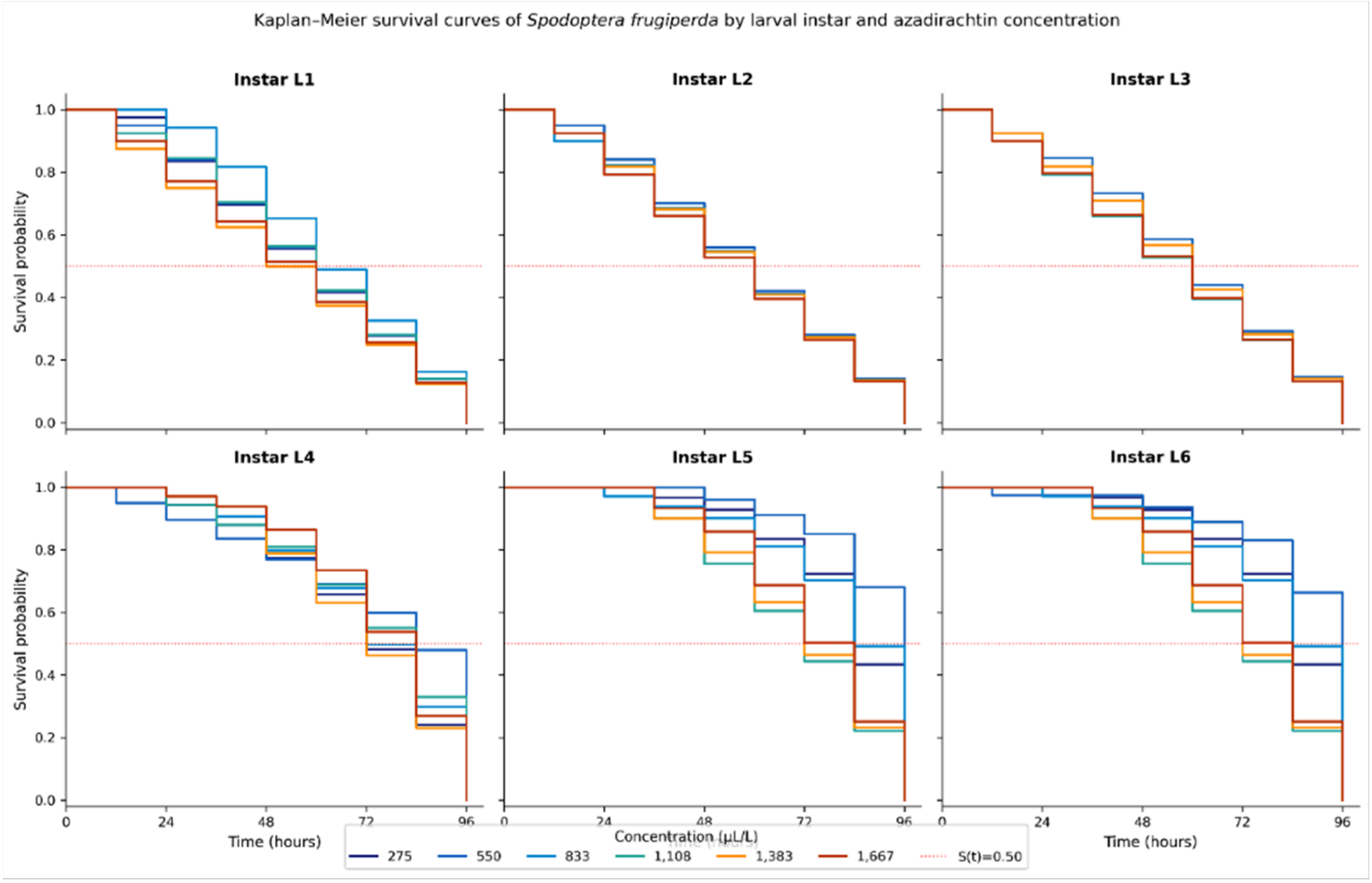


Figure 7

Cox proportional hazards (CPH) forest plot for the two covariates included in the model (larval instar and azadirachtin concentration) (Cohort 2, Phase 2; $\chi^2 = 95.77$, df = 2, $p < 0.001$). HR < 1 indicates a survival-prolonging effect; HR > 1 indicates increased mortality hazard. Exact HR values: instar HR = 0.846 (95% CI: 0.816–0.878); concentration HR = 1.026 (95% CI: 1.013–1.039) per 100 µL/L increment.

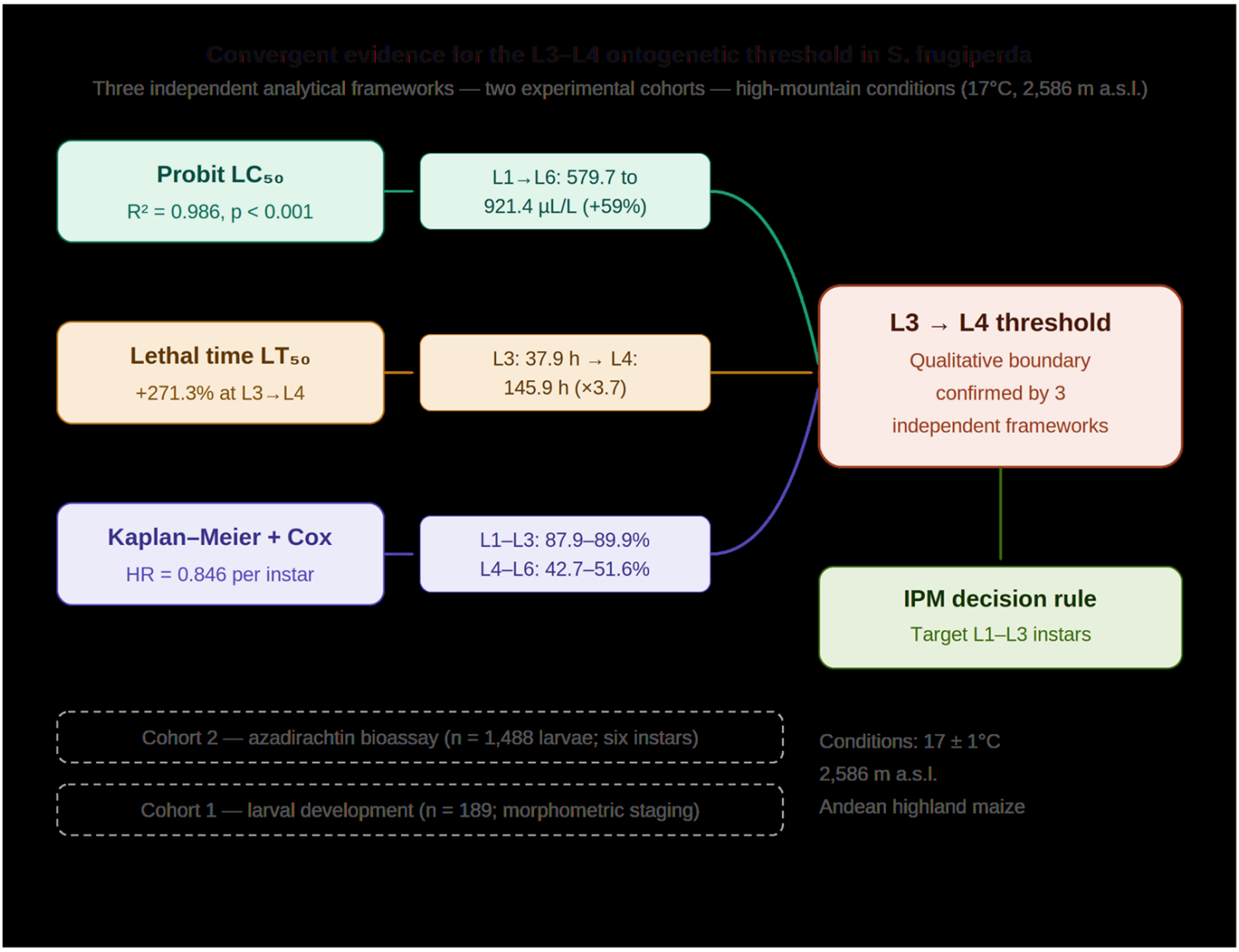


Figure 8

Convergent evidence for the L3–L4 ontogenetic susceptibility threshold in *S. frugiperda* under high-mountain Andean conditions (17 ± 1°C; 2,586 m a.s.l.). Three independent analytical frameworks (Probit LC_{50}, lethal time LT_{50}, and Kaplan–Meier/Cox survival analysis) applied to two experimental cohorts independently converged on the same qualitative boundary between L3 and L4. This threshold defines the IPM decision rule: azadirachtin applications should target L1–L3 instars to maximize efficacy in high-altitude maize agroecosystems.