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

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Abstract

BACKGROUND

Azadirachtin, derived from *Azadirachta indica* A. Juss., is increasingly valued as a botanical insecticide for Integrated Pest Management (IPM) programs. However, most toxicological data on azadirachtin against *Spodoptera frugiperda* (J.E. Smith) have been generated under lowland tropical conditions (20–30°C), leaving a critical knowledge gap regarding its efficacy in Andean high-mountain agroecosystems characterized by cooler temperatures.

RESULTS

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 LC_{50} values below 703 µL/L at 96 h and mean LT_{50} of 43.3 h. A critical physiological threshold between L3 and L4 was identified by convergent evidence from Probit analysis ($R^2 = 0.986$, $p < 0.001$) and lethal time curves, with LT_{50} increasing abruptly by 2.7-fold from L3 to L4 (37.9 vs. 145.9 h). Three-way ANOVA confirmed that larval instar (partial $\eta^2 = 0.399$, $p < 0.001$) and exposure time (partial $\eta^2 = 0.355$, $p < 0.001$) were the dominant factors determining mortality.

CONCLUSION

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 applications: treatments should be timed to target L1–L3 instars to maximize cost-effectiveness and minimize the risk of inconsistent field outcomes. These findings provide the first comprehensive toxicological characterization of azadirachtin against *S. frugiperda* in Andean high-altitude environments.

1 INTRODUCTION

Spodoptera frugiperda (J.E. Smith) (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.[1] 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.[2] Its high reproductive rate, polyphagous feeding behavior, and documented capacity to develop resistance to synthetic insecticides have complicated reliance on conventional chemical management, intensifying interest in sustainable alternatives compatible with Integrated Pest Management (IPM) frameworks.[3]

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 that distinguishes it from conventional insecticides: it acts simultaneously as an antifeedant, an insect growth regulator (IGR), and an endocrine disruptor.[4] 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.[5] Recent studies have further demonstrated that azadirachtin inhibits the nuclear receptor HR3 in the prothoracic gland, thereby blocking larval ecdysis in *Spodoptera frugiperda* at the molecular level.[6] Collectively, these mechanisms result in larval mortality through starvation, failed molting, and developmental arrest — effects that are 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, for example, commonly experience temperatures of 14–18°C throughout the crop cycle — conditions known to reduce insect metabolic rates, extend developmental periods, and potentially alter the uptake and detoxification of ingested compounds.[7] There is also direct evidence that azadirachtin toxicity against other Orthoptera and Lepidoptera is highly temperature-sensitive, with efficacy declining substantially at lower temperatures.[8] This has direct practical implications: if published dose recommendations derived from lowland bioassays are applied uncorrected in highland systems, growers risk suboptimal control outcomes or, conversely, the use of 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*,[9],[10] 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 a critical piece of 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.

2 MATERIALS AND METHODS

2.1 Insect colony and larval rearing

Larvae of *Spodoptera frugiperda* were obtained from a colony maintained at the Agronomic Engineering Laboratory of the Universidad de Pamplona (Pamplona, Norte de Santander, Colombia; 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.[11] Mean developmental durations for each larval instar under these conditions are summarized in Table 1. The prolonged larval period — particularly the sixth instar (mean 13.63 ± 1.75 days) — reflects the characteristic developmental slowing observed in cold-adapted insect populations at high altitude.[12] All larvae used in bioassays were selected at precise ages within each instar to minimize overlap between consecutive developmental stages (Table 1).

2.2 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. Trade names and brands are restricted to the Materials and Methods section per journal policy. Concentrations of the 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 µL/L of commercial product, equivalent to 0 to 20.00 mg/L of technical azadirachtin.

2.3 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 the 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).

2.4 Statistical analyses

Dose–response data at 96 h were analyzed by Probit regression[13] 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 an 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).

3 RESULTS

3.1 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 µL/L of commercial product (equivalent to 8.88 mg/L technical azadirachtin) (Figure 1). Mortality increased sharply at concentrations above 275 µL/L, with all treatments from 275 µL/L onward reaching 100% mortality in L1–L3 at 96 h and near-total mortality (≥99.2%) in all instars (Table 4).

3.2 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 (LC_{50} = 579.7 µL/L; 95% CI: 512.3–655.2), whereas sixth-instar larvae required a 59% higher concentration (LC_{50} = 921.4 µL/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.

3.3 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 µL/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.

3.4 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 × concentration (partial $\eta^2 = 0.063$, $p < 0.001$) and instar × 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 × time was non-significant ($p = 0.249$), suggesting that at the concentrations tested, increasing dose does not substantially accelerate the time course of mortality beyond the instar-determined baseline. The three-way interaction (instar × concentration × time) was also non-significant ($p = 0.953$), consistent with a model in which developmental stage is the primary modulator of azadirachtin efficacy.

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 obtained in this study is consistent with the range reported in Brazilian studies under lowland conditions (600–1,200 µL/L for similar commercial formulations),[14,15] 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,[8] 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.[6] 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.[5] In contrast, fifth- and sixth-instar larvae

have progressively committed to metamorphosis preparation and may rely less on continuous PTTH 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,[9] attributed to increased body mass, cuticular thickening, and upregulation of detoxification enzymes including cytochrome P450s.[10] The present data suggest 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. Notably, the concurrent companion study on *Eiphosoma vitticolle* Cresson parasitizing *S. frugiperda* in the same agroecosystem identified the same L3–L4 boundary as the critical susceptibility window for parasitoid acceptance and immune encapsulation — suggesting that this instar transition marks a broadly important physiological reorganization in *S. frugiperda* that is independently detectable by both chemical and biological methods.

The three-way ANOVA result that larval instar (partial η^2 = 0.399) accounts for more variance in mortality than either concentration (partial η^2 = 0.049) or time (partial η^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.[3,16] 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 explicitly 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 like *Eiphosoma vitticolle* may be more appropriate for managing late-instar populations in these environments.[17]

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.[14] Future work should compare purified azadirachtin with full-spectrum neem formulations under high-altitude conditions to disentangle these contributions.

5 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.

Declarations

Ethical Approval

This study did not involve human participants or vertebrate animals. Research was conducted using laboratory-reared Spodoptera frugiperda (Lepidoptera: Noctuidae) under controlled conditions. Ethical approval is not applicable.

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Authors' Contributions

H.G.-V. conceived and designed the research, developed the experimental protocol, performed all statistical analyses, interpreted the results, and is solely responsible for the intellectual content and original writing of this manuscript. M.Y.R.-P. and Z.V.C.-M., as undergraduate members of the Semillero de Investigación en Sanidad Vegetal Sustentable, maintained the Spodoptera frugiperda laboratory colony, assisted in bioassay execution, recorded experimental data, and contributed to data curation and figure preparation under the direct supervision of H.G.-V. All authors reviewed and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Developmental parameters of larval instars of Spodoptera frugiperda under laboratory conditions (17 ± 1°C, 65 ± 10% RH) and selected ages for bioassays.

Instar	Mean duration (days ± 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 Núñez-García et al.[11] Selected ages were defined to minimize overlap between consecutive instars given natural developmental variability.

Table 2. Neem oil concentrations evaluated in the toxicological bioassay against Spodoptera frugiperda under laboratory conditions (1.2% EC formulation; 12 g azadirachtin/L).

Treatment	Commercial product concentration (mL/L)	Applied azadirachtin (µL/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 Spodoptera 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**

Table 4. Total mortality (%) caused by different neem oil concentrations on six larval instars of Spodoptera frugiperda under laboratory conditions at 96 h of exposure.

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

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 neem oil for each larval instar of Spodoptera frugiperda at 96 h of exposure.

Instar	Probit regression 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

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

Table 6. Median lethal time (LT_{50}), Probit regression equations, and coefficients of determination (R^2) for first-instar larvae of Spodoptera frugiperda exposed to different neem oil concentrations.

Concentration (µL/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 (LT_{50}), Probit regression equations, and coefficients of determination (R^2) for second-instar larvae of Spodoptera frugiperda exposed to different neem oil concentrations.

Concentration (µL/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 (LT_{50}), Probit regression equations, and coefficients of determination (R^2) for third-instar larvae of Spodoptera frugiperda exposed to different neem oil concentrations.

Concentration (µL/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 (LT_{50}), Probit regression equations, and coefficients of determination (R^2) for fourth-instar larvae of Spodoptera frugiperda exposed to different neem oil concentrations.

Concentration (µL/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 (LT_{50}), Probit regression equations, and coefficients of determination (R^2) for fifth-instar larvae of Spodoptera frugiperda exposed to different neem oil concentrations.

Concentration (µL/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 (LT_{50}), Probit regression equations, and coefficients of determination (R^2) for sixth-instar larvae of Spodoptera frugiperda exposed to different neem oil concentrations.

Concentration (µL/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.

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

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 (effect size); R^2 = 0.613; adjusted R^2 = 0.660.

Figures

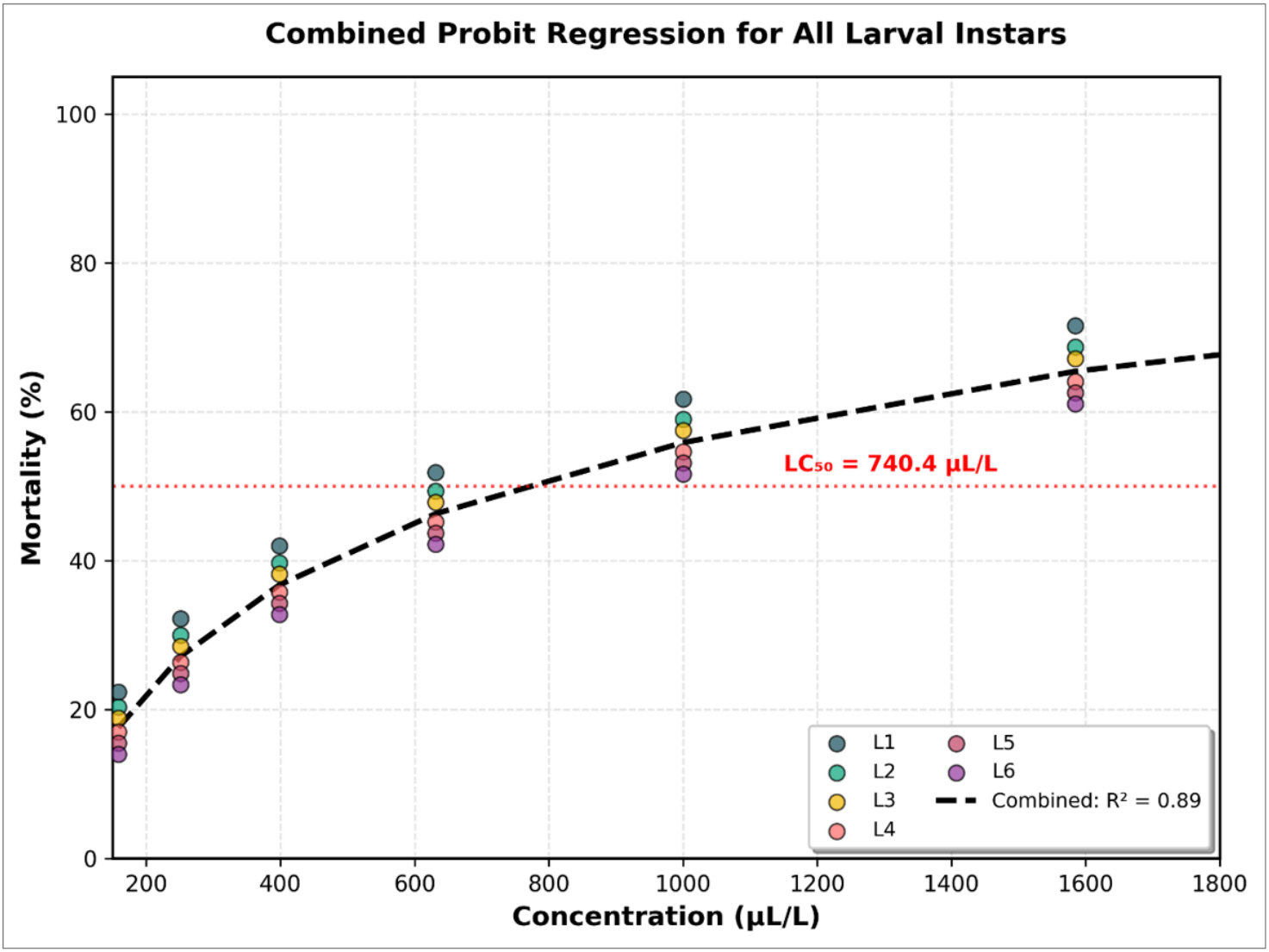

Figure 1

Combined Probit regression for all larval instars of *Spodoptera 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 µL/L (R^2 = 0.89) is indicated. Points are color-coded by larval instar (L1–L6).

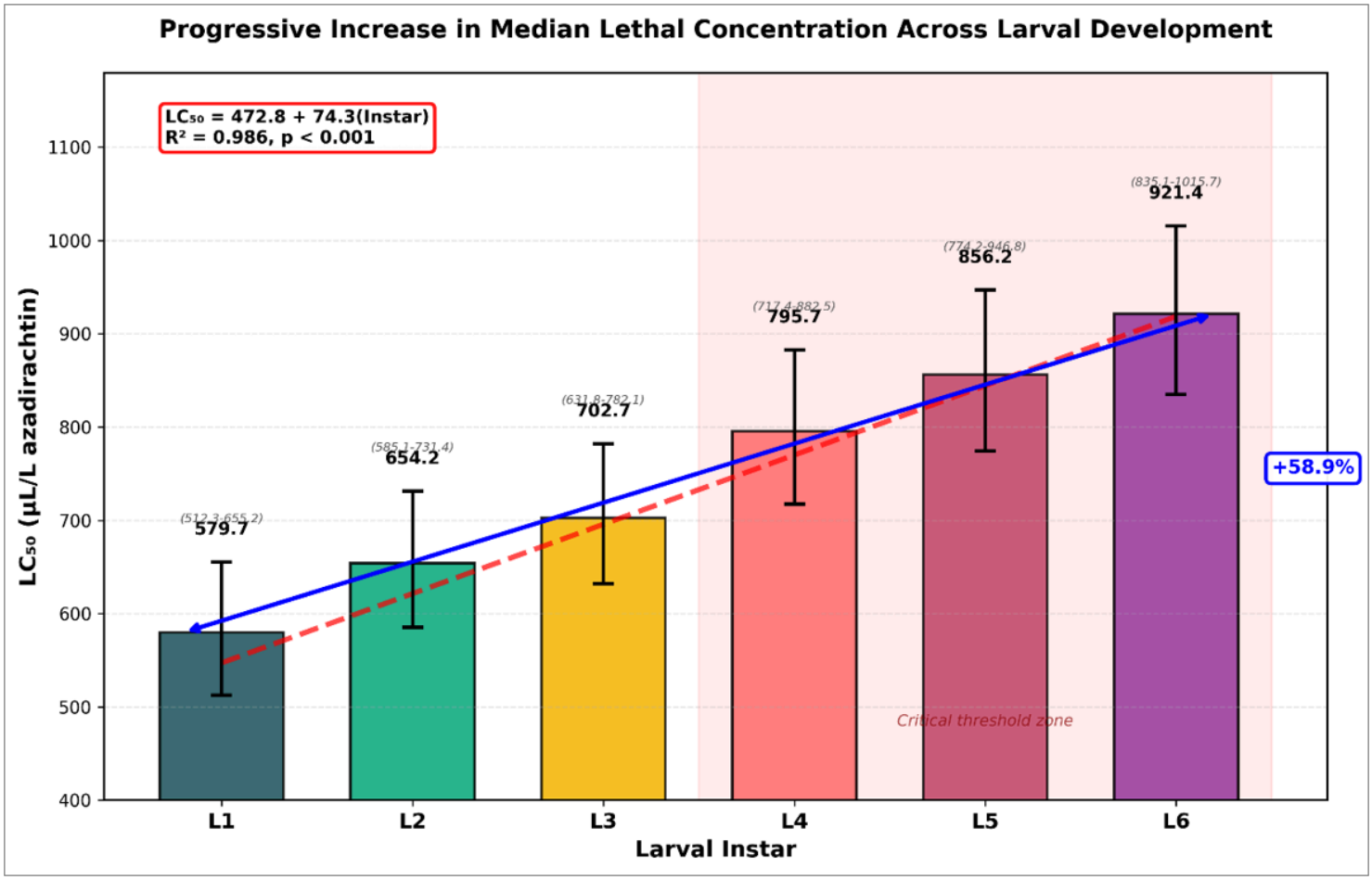

Figure 2

Progressive increase in median lethal concentration (LC_{50}) 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 ($R^2 = 0.986$, $p < 0.001$; $LC_{50} = 472.8 + 74.3 \times$ instar). The total increase from L1 to L6 (+58.9%) is annotated.

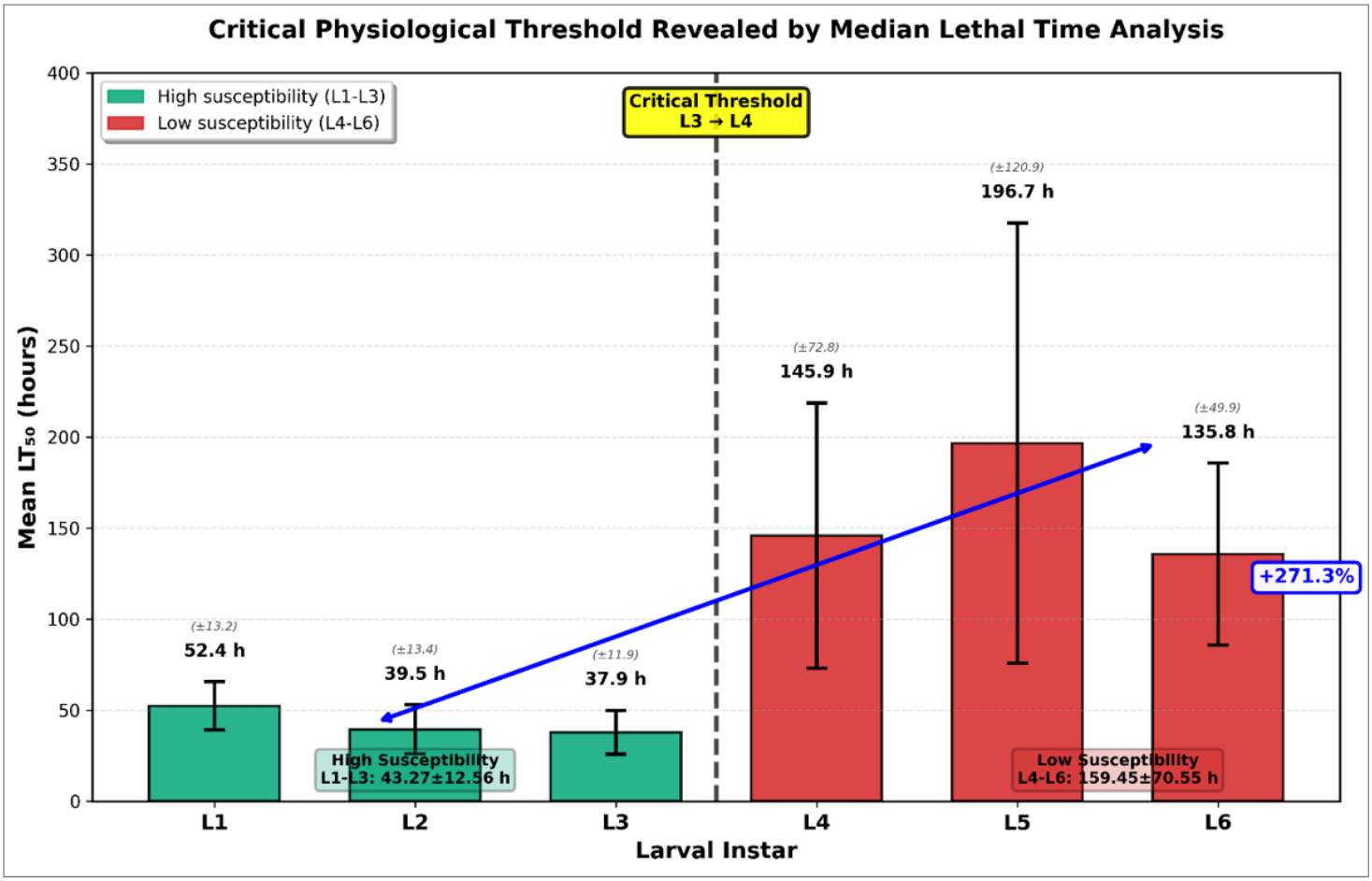

Figure 3

Critical physiological threshold revealed by mean LT_{50} analysis across larval instars of *S. frugiperda*. Green bars represent early instars (L1–L3; mean LT_{50} = 43.27 ± 12.56 h); red bars represent late instars (L4–L6; mean LT_{50} = 159.45 ± 70.55 h). The dashed vertical line marks the L3→L4 threshold, corresponding to a 271.3% increase in mean LT_{50}. Error bars represent standard deviation across concentrations.

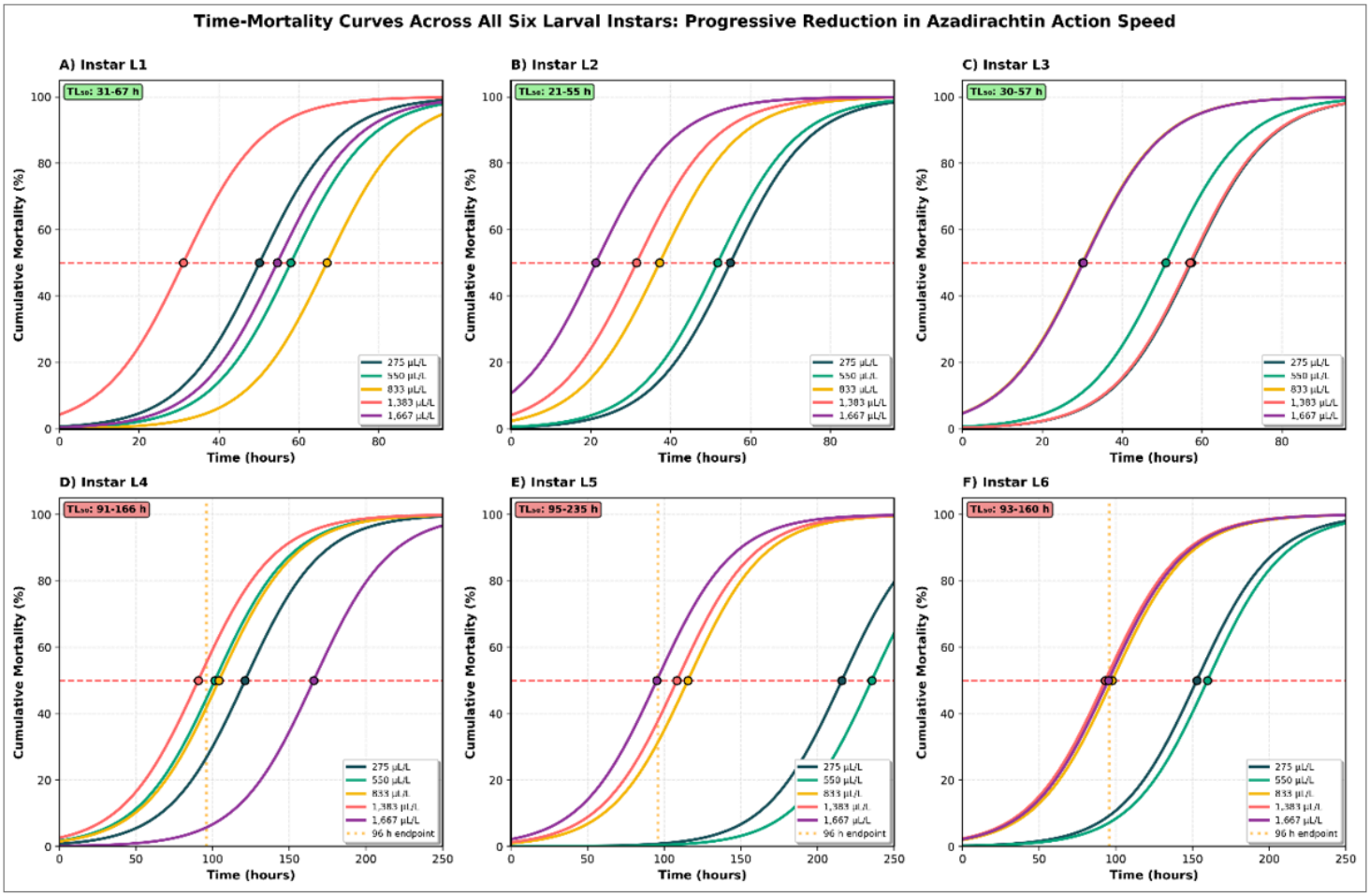

Figure 4

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 neem oil concentration (275–1,667 µL/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. LT_{50} ranges are annotated for each panel.