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RESEARCH ARTICLE

# The Scar of Rebuilding: Limb Regeneration Triggers Asymmetric Melanisation and Developmental Trade-Offs

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## ABSTRACT

Melanisation is a key insect immune response activated by injury, yet its role as a long-term indicator of regeneration costs is unclear. This study examined whether limb regeneration in the ladybird beetle (*Cheilomenes sexmaculata*) leads to persistent immune investment, seen as increased cuticular melanisation, and what trade-offs emerge. Larvae amputated in each instar showed that regeneration triggered a systemic immune response, with significantly elevated melanisation specifically on the contralateral elytra. This was accompanied by prolonged development and, after fourth-instar amputation, larger elytra size. These findings reveal that regeneration imposes organism-wide costs, permanently altering immune status, development, and morphology, with melanisation serving as a visible signature of these physiological trade-offs.

**KEY WORDS:** Melanisation, Immune trade-off, Developmental cost, Compensatory growth, *Cheilomenes sexmaculata*, Limb regeneration

## INTRODUCTION

Melanisation represents a fundamental and visually striking biological process in insects, serving dual roles in immune defence and cuticular pigmentation (Cerenius & Söderhäll, 2004; Nappi & Christensen, 2005; Gonzalez-Santoyo & Cordoba-Aguilar, 2012). As a key immune response, it involves the rapid synthesis of melanin at sites of infection or injury to encapsulate pathogens and facilitate wound healing (Cerenius *et al.,* 2008). Concurrently, melanism is one of the most conspicuous forms of morphological variation, observed across insect species (Majerus, 1998; Joron *et al.,* 2006; Wittkopp & Beldade, 2009), between populations of the same species (Safranek & Riddiford, 1975; Bear *et al.,* 2010), and among individuals within a population (Thompson *et al.,* 2002). This variation can be expressed locally on specific body regions, as is common in Diptera (Brisson *et al.,* 2005; Singh *et al.,* 2009) and Coleoptera (Michie *et al.,* 2010; Brakefield & de Jong, 2011), or globally across the entire body.

The evolution of melanism is driven by diverse selective forces and carries inherent fitness trade-offs. Benefits can include faster development and higher fecundity, as observed in *Mythimna separata* Walker (Jiang *et al.,* 2007) and *Malacosoma disstria* Hübner (Lorimer, 1979), and faster development in *Biston betularia* Linnaeus (Grant & Clarke, 1999). Conversely, costs can manifest as lower survival, fecundity, body weight, and slower development, as documented in *Manduca sexta* Linnaeus (Safranek & Riddiford, 1975), *Helicoverpa armigera* Hübner (Ma *et al.,* 2008), and *Bicyclus anynana* Butler (Bear *et al.,* 2010). In ladybirds, the study of this polymorphism has a long history, initially used for taxonomy and later for genetics, with early work suggesting control by multiple alleles at a single locus and later revelations of genetically linked loci for winglessness and elytral melanism in *Adalia bipunctata* Linnaeus (Lommen *et al.,* 2005,2012). The ecological significance and spatiotemporal distribution of these morphs have been extensively studied (Honek, 1975; Majerus & Zakharov, 2000; Honek *et al.,* 2005; Wang *et al.,* 2009; Michie *et al.,* 2010, 2011; Brakefield & de Jong, 2011).

The melanisation cascade, mediated by phenoloxidase, represents a rapid and evolutionarily conserved immune mechanism in insects, providing a

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direct functional link between physical injury and immune activation. This response is well-documented across diverse orders, including Blattodea (*Periplaneta Americana* Linnaeus), Coleoptera (*Tenebrio molitor* Linnaeus, *Tribolium castaneum* Herbst), Diptera (*Anopheles gambiae* Giles, *Drosophila melanogaster* Meigen), Lepidoptera (*Bombyx mori* Linnaeus, *Manduca sexta* Linnaeus), and Hymenoptera (*Apis mellifera* Linnaeus) (Marieshwari *et al.,* 2023). Upon wounding, the prophenoloxidase cascade is activated, leading to melanin deposition at injury sites to block pathogen invasion and promote tissue repair, a process observed within minutes in *D. melanogaster* (Dudzic *et al.,* 2015). This critical role is further exemplified in *Galleria mellonella* Linnaeus, where intense melanisation encapsulates and neutralizes pathogens (Smith *et al.,* 2022).

This intimate link between injury and immunity is intrinsically connected to the process of regeneration, the ability to replace lost body parts, which must be integrated within the rigid exoskeleton and hormone-driven development of insects (Mito *et al.,* 2002; Bely & Nyberg, 2010). As a metabolically costly process, regeneration creates trade-offs with other life-history traits like growth and development (Maiorana, 1977; Bulliere, 1985; Lawrence & Larrain, 1994; Juanes & Smith, 1995). Within this framework, the melanisation cascade constitutes an essential component of the integrated repair response, facilitating cuticle sclerotization and wound sealing through melanin deposition (Nappi & Christensen, 2005; Gonzalez-Santoyo & Cordoba-Aguilar, 2012). Thus, melanisation forms a crucial biological bridge connecting initial tissue damage, immediate immune defence, and the subsequent process of regenerative repair.

In the ladybird beetle *Cheilomenes sexmaculata* (Fabricius) (Coleoptera: Coccinellidae), larval appendage loss can be regenerated in adulthood, though the resulting legs are often smaller (Saxena *et al.,* 2016; Rai *et al.,* 2023; Alam *et al.,* 2024). While previous research has established that limb regeneration affects development (Wu *et al.,* 2019a), morphometry (Wu *et al.,* 2019b; Rai *et al.,* 2023; Alam *et al.,* 2024), and functional recovery (Yang *et al.,* 2016), its specific influence on the melanic immune response remains underexplored. Therefore, this study aims to bridge this gap by investigating the interplay between regeneration and melanisation. We hypothesized that limb regeneration in *C. sexmaculata* larvae induces a systemic immune response, manifesting as increased melanisation in the adult cuticle. The primary objective is to quantify this cost of regeneration by analysing the percent melanisation of the elytra as a marker of persistent immune activation.

## METHODOLOGY

### Insect Collection and Rearing

Approximately 80 adult *C. sexmaculata* beetles were collected from cowpea (*Vigna unguiculata* (L.) Walp.) fields in Lucknow, India (26.8467° N, 80.9462° E). The stock culture was maintained in a controlled environment room (27 ± 2°C, 65 ± 5% R.H., 14L:10D photoperiod) in large plastic beakers covered with muslin cloth. Beetles were provisioned daily with the cowpea aphid, *Aphis craccivora* Koch, which were reared on cowpea plants in a polyhouse under similar conditions. To ensure acclimatization, the beetles were reared for two generations in the laboratory before experimentation. For egg collection, mated females were transferred to Petri dishes (9 × 2 cm) with *ad libitum* aphids. The eggs were collected and held until eclosion, after which individual neonates were reared in separate Petri dishes and provided with aphids daily.

### Experimental Design and Amputation Procedure

Newly hatched larvae were randomly divided into five treatment groups (n=20 per group). Four groups underwent amputation of the right foreleg at the proximal femur within 24 hours of moulting to a specific instar: first, second, third, or fourth. A fifth group served as an unamputated control. All amputations were performed under a stereomicroscope using a fine micro-scalpel. The amputated and control larvae were reared individually under the standard laboratory conditions described above and monitored daily until adult emergence to record developmental durations: pupal duration (PD), post-amputation developmental duration (PADD), and total developmental duration (TDD).

### Elytra Measurement and Morphometric Analysis

Following a 24-hour post-emergence period to allow for cuticular sclerotization, the elytra were carefully separated from the adult beetles using fine forceps. Digital images were captured under a stereoscopic dissecting microscope (2.5× magnification) and analysed using ImageJ software (v1.53u). A standardized scale was used for calibration. The length, area, and perimeter of each elytron were then measured, and the area of melanised regions in binary image were calculated to quantify the percent melanisation.

### Statistical Analysis

Data distribution was assessed using the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances. The measurements for left and right elytra length, area, and perimeter conformed to a normal distribution. In contrast, the total developmental

duration (TDD), pupal duration (PD), post-amputation developmental duration (PADD), and the percent melanisation of both left and right elytra deviated from normality.

To evaluate the impact of limb regeneration on each trait, separate Generalized Linear Models (GLMs) were implemented. A linear model with an identity link function was used for normally distributed data, while a gamma distribution with a log link function was applied to non-normal data. Treatment was included as a fixed factor in all models. All analyses were conducted in IBM SPSS Statistics (v26.0), with a significance level of $\alpha = 0.05$.

## RESULTS

### Larval Amputation Alters Developmental Duration

The amputation treatment had a significant effect on the pupal duration (Wald $\chi^2 = 62.959$, df = 4, $P < 0.001$). Post-hoc comparisons revealed that the pupal duration of control adults was significantly shorter than that of regenerated adults amputated at the second (Mean difference = -0.85 ± 0.171, df = 1, $P < 0.001$), third (Mean difference = -0.60 ± 0.164, df = 1, P = 0.003), and fourth (Mean difference = -1.20 ± 0.181, df = 1, $P < 0.001$) larval instars (Fig. 1a) (Table 1).

Both treatment and larval stage at amputation significantly influenced the post-amputation development

Table 1: Result of univariate analysis on dependent variables (pupa duration, total development duration and post amputation development duration) with treatment (control and amputation at different larval instars) as independent factor ($\alpha$=0.05). Mean±SE pupa duration, total development duration and post amputation development duration of control and regenerated individuals. First, second, third and fourth represents the larval stage of amputation whereas control represents the normal adults without any amputation.

| Amputation stages | Pupa duration (days) | Total development duration (days) | Post amputation development duration (days) | |
|---|---|---|---|---|
| Control | 2.95±0.105 | 14.70±0.230 | Control | Regenerated |
| First larval instar | 3.10±0.110 | 17.20±0.269 | 11.70±0.235 | 14.20±0.235 |
| Second larval instar | 3.80±0.135 | 16.55±0.259 | 10.55±0.235 | 12.45±0.235 |
| Third larval instar | 3.55±0.126 | 15.95±0.250 | 9.000±0.235 | 9.950±0.235 |
| Fourth larval instar | 4.15±0.148 | 15.45±0.242 | 6.850±0.235 | 8.150±0.235 |
| F, df, P-Value | Wald $\chi^2 = 62.959$, df = 4, $P < 0.001$ | Wald $\chi^2 = 60.164$, df = 4, $P < 0.001$ | Treatment: Wald $\chi^2 = 121.160$, df = 1, $P < 0.001$; Stage: Wald $\chi^2 = 824.100$, df = 3, $P < 0.001$ | |

Table 2: Result of univariate analysis on dependent variables (Elytra melanisation, length, area and perimeter) with treatment (Unamputated and amputated at different larval instars) as independent factor ($\alpha$=0.05). Mean±SE melanisation, length, area and perimeter of control and regenerated adult foreleg. First, second, third and fourth represents the larval stage of amputation whereas control represents the normal adults without any amputation.

| Amputation | Elytra melanisation (%) | | Elytra length (mm) | | Elytra area (mm²) | | Elytra perimeter (mm) | |
|---|---|---|---|---|---|---|---|---|
| stages | Left | Right | Left | Right | Left | Right | Left | Right |
| Control | 26.18±1.271 | 27.79±1.403 | 3.736±0.073 | 3.739±0.074 | 7.210±0.307 | 7.755±0.281 | 10.16±0.201 | 10.52±0.181 |
| First larval instar | 31.78±1.543 | 31.03±1.567 | 3.505±0.073 | 3.507±0.074 | 7.554±0.307 | 7.573±0.281 | 10.33±0.201 | 10.31±0.181 |
| Second larval instar | 31.82±1.545 | 31.58±1.594 | 3.581±0.073 | 3.584±0.074 | 8.080±0.307 | 7.916±0.281 | 10.71±0.201 | 10.68±0.181 |
| Third larval instar | 32.80±1.593 | 31.58±1.594 | 3.505±0.073 | 3.508±0.074 | 7.194±0.307 | 7.170±0.281 | 10.16±0.201 | 10.23±0.181 |
| Fourth larval instar | 33.12±1.608 | 33.62±1.698 | 3.697±0.073 | 3.700±0.074 | 8.734±0.307 | 8.740±0.281 | 11.14±0.201 | 11.24±0.181 |
| F, df, P-Value | Left: Wald $\chi^2 = 15.842$, df = 4, P = 0.003 | | Left: Wald $\chi^2 = 8.436$, df = 4, P = 0.077 | | Left: Wald $\chi^2 = 18.199$, df = 4, P = 0.001 | | Left: Wald $\chi^2 = 17.841$, df = 4, P = 0.001 | |
| | Right: Wald $\chi^2 = 7.649$, df = 4, P = 0.105 | | Right: Wald $\chi^2 = 8.440$, df = 4, P = 0.077 | | Right: Wald $\chi^2 = 16.907$, df = 4, P = 0.002 | | Right: Wald $\chi^2 = 19.640$, df = 4, P = 0.001 | |

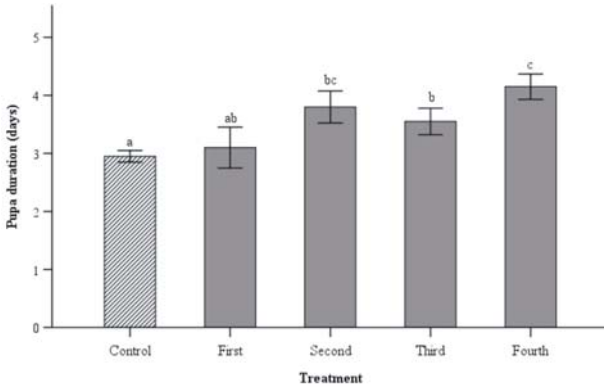


Fig. 1a

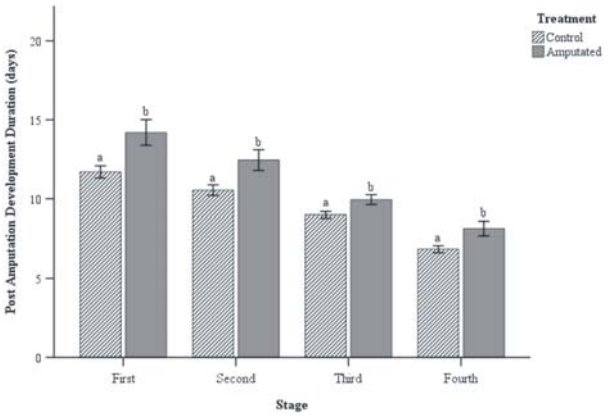


Fig. 1b

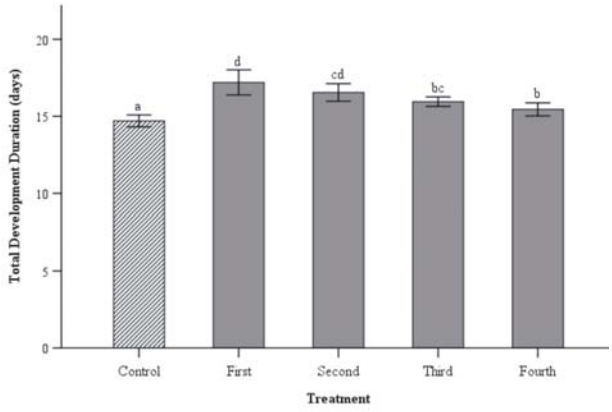


Fig. 1c

Fig. 1. Effect of larval stage of amputation on developmental transitions of *C. sexmaculata* adults. (a) Pupa (b) Post amputation development duration (c) Total development duration. Values are mean ± SE. First, Second, Third and Fourth represents the larval stage of amputation. Similar letters indicate a lack of significant difference at P > 0.05.

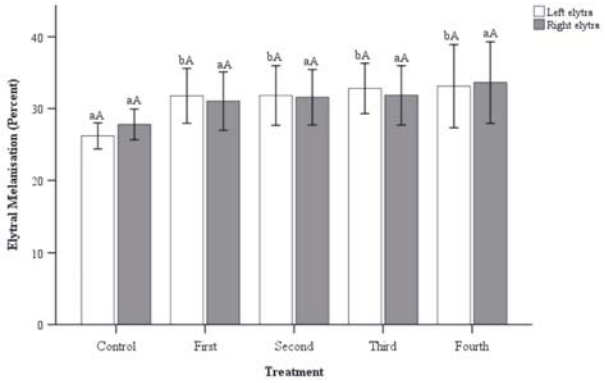


Fig. 2. Effect of larval stage of amputation on elytra melanisation (in percent) of *C. sexmaculata* adults. Values are mean ± SE. Lowercase and uppercase letters indicate the comparison of means across and within the treatments, respectively. First, Second, Third and Fourth represents the larval stage of amputation. Similar letters indicate a lack of significant difference at P > 0.05.

duration (PADD) (Treatment: Wald $\chi^2 = 121.160$, df = 1, P < 0.001; Stage: Wald $\chi^2 = 824.100$, df = 3, P < 0.001). The PADD was significantly prolonged in regenerated adults compared to the control, regardless of the instar at which amputation occurred: first (Mean difference = 2.50 ± 0.374, df = 1, P < 0.001), second (Mean difference = 1.90 ± 0.332, df = 1, P < 0.001), third (Mean difference = 0.95 ± 0.273, df = 1, P = 0.014), and fourth (Mean difference = 1.30 ± 0.217, df = 1, P < 0.001) (Fig. 1b) (Table 1).

Consequently, the total development duration (TDD) also differed significantly across treatments (Wald $\chi^2 = 60.164$, df = 4, P < 0.001). The TDD of regenerated adults was prolonged compared to controls when amputation was performed at the first (Mean difference = 2.50 ± 0.354, df = 1, P < 0.001), second (Mean difference = 1.85 ± 0.346, df = 1, P < 0.001), third (Mean difference = 1.25 ± 0.339, df = 1, P = 0.002), and fourth (Mean difference = 0.75 ± 0.334, df = 1, P = 0.046) larval instars (Fig. 1c) (Table 1).

## Melanisation is Affected in Regenerated Elytra

A significant treatment effect was observed for the percent melanisation on the left elytron (Wald $\chi^2 = 15.842$, df = 4, P = 0.003). The melanisation percentage in control adults was significantly lower than in regenerated adults amputated at the first (Mean difference = -5.595 ± 1.999, df = 1, P = 0.045), second (Mean difference = -5.634 ± 2.001, df = 1, P = 0.049), third (Mean difference = -6.618 ± 2.038, df = 1, P = 0.012), and fourth (Mean difference = -6.936 ± 2.050, df = 1, P = 0.007) instars. No significant differences in melanisation were detected among the different regeneration treatment groups. In contrast, percent

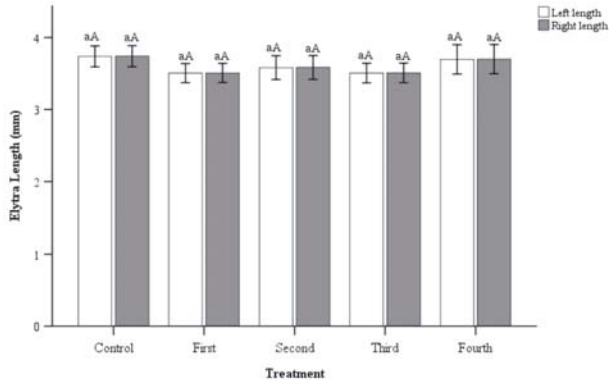


Fig. 3a

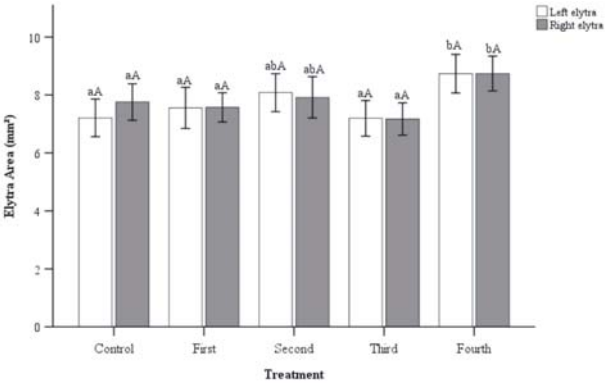


Fig. 3b

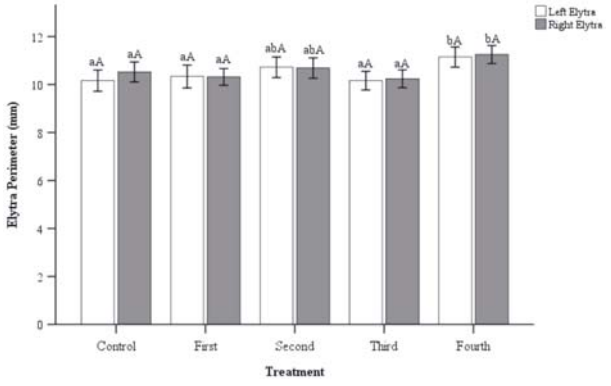


Fig. 3c

Fig. 3. Effect of larval stage of amputation on elytra dimensions of *C. sexmaculata* adults. (a) Elytra length (b) Elytra area (c) Elytra perimeter. Values are mean ± SE. Lowercase and uppercase letters indicate the comparison of means across and within the treatments, respectively. First, Second, Third and Fourth represents the larval stage of amputation. Similar letters indicate a lack of significant difference at $P > 0.05$.

melanisation on the right elytron did not differ significantly across treatments (Wald $\chi^2 = 7.649$, df = 4, P = 0.105), and no differences were found between the right and left elytra within treatments (Fig. 2) (Table 2).

## Elytra Morphology Following Regeneration

The length of both left and right elytra did not differ significantly across treatments (Left: Wald $\chi^2 = 8.436$, df = 4, P = 0.077; Right: Wald $\chi^2 = 8.440$, df = 4, P = 0.077) (Fig. 3a) (Table 2). However, the area of the left elytron was significantly affected by treatment (Wald $\chi^2 = 18.199$, df = 4, P = 0.001). The left elytra area of adults regenerated after amputation at the fourth instar was significantly larger than that of controls (Mean difference = 1.524 ± 0.434, df = 1, P = 0.004), as well as those amputated at the first (Mean difference = 1.180 ± 0.434, df = 1, P = 0.043) and third instars (Mean difference = 1.539 ± 0.434, df = 1, P = 0.004). No other pairwise differences were significant (Fig. 3b) (Table 2). Similarly, the area of the right elytron varied significantly across treatments (Wald $\chi^2 = 16.907$, df = 4, P = 0.002). Adults regenerated after fourth-instar amputation had a larger right elytron area than controls (Mean difference = 0.984 ± 0.398, df = 1, P = 0.045) and those amputated at the first (Mean difference = 1.166 ± 0.398, df = 1, P = 0.034) and third instars (Mean difference = 1.569 ± 0.398, df = 1, P = 0.001). No significant difference was observed between the areas of the right and left elytra across all treatments (Fig. 3b) (Table 2).

The perimeter of the left elytron showed significant variation among treatments (Wald $\chi^2 = 17.841$, df = 4, P = 0.001). The left elytron perimeter was significantly larger in adults regenerated after fourth-instar amputation compared to controls (Mean difference = 0.984 ± 0.284, df = 1, P = 0.005) and those amputated at the first (Mean difference = 0.812 ± 0.284, df = 1, P = 0.043) and third instars (Mean difference = 0.984 ± 0.284, df = 1, P = 0.005) (Fig. 3c) (Table 2). A significant treatment effect was also found for the right elytron perimeter (Wald $\chi^2 = 19.640$, df = 4, P = 0.001). It was larger in the fourth-instar amputation group than in controls (Mean difference = 0.725 ± 0.255, df = 1, P = 0.046) and the first (Mean difference = 0.930 ± 0.255, df = 1, P = 0.003) and third-instar groups (Mean difference = 1.008 ± 0.255, df = 1, P = 0.001). No significant differences were detected between the perimeters of the right and left elytra for any treatment (Fig. 3c) (Table 2).

## DISCUSSION

This study demonstrates that limb amputation and subsequent regeneration in larvae significantly alter key developmental and morphological traits in the resulting adult ladybird beetles. The observed delays in development, the asymmetric increase in elytral

melanisation, and the instar-specific changes in elytra size collectively indicate that the regenerative process imposes substantial physiological costs with lasting consequences.

The most pronounced effect was a significant prolongation of the total development duration, primarily driven by an extended post-amputation development duration (PADD). This period encompasses the time required for wound healing and limb bud regeneration before normal development can resume. Our findings align with the fundamental principle of resource allocation trade-offs, where finite energetic resources are diverted from growth and maturation towards the costly process of regeneration (Maginnis, 2006; Bely & Nyberg, 2010). Similar developmental delays have been documented in other regenerating insects (Dewes, 1973; Kunkel, 1977; Wang *et al.,* 2015; Wu *et al.,* 2019a; Alam *et al.,* 2024; Rai *et al.,* 2024). This delay is likely a systemic stress response, where development is actively halted until damaged tissue is repaired, ensuring the emergence of a morphologically normal adult (Madhavan & Schneiderman, 1969; Smith-Bolton *et al.,* 2009). The underlying mechanism may involve a disruption in ecdysteroid signalling, a key hormone governing developmental transitions (Riddiford & Truman, 1993; Yamanaka *et al.,* 2013), providing a direct explanation for the observed delay (Shingleton *et al.,* 2005; Garelli *et al.,* 2012). This suggests the delay is an active, hormonally regulated adaptation to injury.

A key morphological consequence of regeneration was the elevated melanisation observed specifically on the left elytra. Melanisation is a cornerstone of the insect innate immune system, involved in wound healing, sclerotization, and pathogen encapsulation (Ashida & Brey, 1997; Sugumaran, 2002; Cerenius & Söderhäll, 2004; Kanost *et al.,* 2004; Nappi & Christensen, 2005; Gonzalez-Santoyo & Cordoba-Aguilar, 2012). The observed pigmentation likely results from a systemic immune and endocrine response to the combined trauma of injury and the sustained metabolic demand of regeneration. This localized increase suggests a persistent, systemic immune response, consistent with studies showing that injury sustains phenoloxidase activation (Liu *et al.,* 2007; Nappi *et al.,* 2009; Binggeli *et al.,* 2014). The absence of this effect on the ipsilateral (right) elytron was unexpected. This asymmetry may indicate a resource allocation trade-off where the injury site prioritizes regeneration, limiting local melanisation, while the contralateral elytra manifests the systemic immune signal more visibly. The consistency of this effect across all instars confirms it is a fundamental consequence of the regeneration event itself.

Interestingly, while elytra length remained unaffected among all treatments, the area and perimeter of the elytra were significantly larger in adults that regenerated a limb after amputation in the fourth instar. This points to a critical developmental window where the hormonal environment is susceptible to morphological change. The regenerative process may have triggered systemic shifts in hormones like juvenile hormone or ecdysone (Nijhout, 1994; Truman & Riddiford, 2002), causing over-proliferation of epidermal cells and leading to larger elytra dimensions without altering their basic form.

In conclusion, our findings provide strong evidence that regenerating a limb is not a neutral process but one that carries significant, organism-wide trade-offs. The process diverts energy, causing developmental delays, and triggers a complex physiological response that permanently alters both the immune status and morphology of the adult. The instar-specific effects on morphology highlight the importance of developmental timing in shaping regenerative outcomes. Future studies should quantify the energetic budget of regeneration and its associated immune response, specifically profiling how hormonal shifts coordinate both melanisation and tissue regrowth to shape the adult insect.

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