Limb regeneration is blastema dependent in a ladybird beetle, *Cheilomenes Sexmaculata* (Fabricius)

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Authors Contributions

SR and GM conceived the presented idea and designed the experiment; SR and SaR collected the sample and conducted the experiment; SaR analysed the data; GM and SR supervised the complete study and along with SR contributed in writing of the manuscript. All authors contributed significantly to the draft and gave final approval for publication.

Conflict of Interest

All authors are in agreement with the content of the manuscript and have no conflict of interest.

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Abstract

Holometabolous insects undergoes metamorphosis which involves an intercalated pupa stage between larva and adult. The body plan of the adult is established during pupa stage and larval systems are de-differentiated and reorganized in insects undergoing complete metamorphosis. In ladybird beetles, the limbs amputated in the larval stages are regenerated in the adults. This occurs during pupation. Given that changes in the pupa are akin to embryogenesis, does the lost limbs are redeveloped as a part of metamorphosis or has some pre-patterning initiated prior pupation? To study this we hypothesised that limb regeneration in a holometabolous ladybird beetles, *Cheilomenes sexmaculata* is the result of the recapitulation of the embryonic gene programs in the pupa. To test this, we exposed the third larval stages of *Cheilomenes sexmaculata* (Fabricius) to amputation and prior to amputation the tissues on the amputated site was scraped off every 24hrs. It was found that limb regeneration does not occur in the treatment where scrapping was done. Assuming that these epidermal cells correspond to blastema, for limb regeneration in ladybird beetles blastema is essential and does not occur in its absence.

Keywords: Amputation; leg development; holometabolous; pupa; metamorphosis

Introduction

Insects form the most diverse group of animals, potentially including 90% of the total animal life existing on earth. Engel and Grimaldi (2005), reported that more than 80% of the insects undergoes metamorphosis which involves a n intercalated pupal stage between larva and adult. The transformation of larval body into conspicuously different adult is referred to as complete metamorphosis or holometaboly. Mostly immobile, there is extensive remodelling of organs and tissues in pupal stage resulting in rebuilding of entire body plan (Rolff et al., 2019). Also, there is no active feeding during metamorphosis. Few structures absent in larval stages appear in the adult and perhaps the signals for these future organs rest in the pupal stage. Altogether, these physiological properties along with undergoing processes resemble embryogenesis.

These signals get activated during metamorphosis and are responsible for the development of structures at stereotypical positions in adult. Pupal stage involves the whole-body reorganization; most larval tissues and organs are respecified including epidermis (Riddiford 1978) and muscles (Lubischer et al 1999). Certain cells with latent embryonic potential are arrested during larval development. These cells initiate to differentiate during pupation forming imaginal primordia, contributing to the development of adult structures and maintain pluripotency similar to stem cells (Truman and Riddiford, 2002; McClure and Schubiger, 2007). Therefore, it is a crucial life stage of holometabolous insects.

Genes associated with metabolism and development, change their level of expression during larval stage and goes back to embryonic-like state during metamorphosis. Ozerova and Gelfand (2022), hypothesised that there is recapitulation of the embryonic expression program during metamorphosis. By the comprehensive analyses of various datasets of holometabolous insects, they demonstrated an increased similarity of pupa to embryonic stage. In another study

on the midge *Polypedilum vanderplanki*, transcriptome profile showed similar reversion to embryonic stage during metamorphosis (Mazin et al., 2018).

Of all the holometabolous insects that undergo regeneration, For example, Lepidoptera (Yang et al. 2016) and Coleoptera (Wang et al. 2015, Saxena et al. 2016, Michaud et al., 2020) the amputated part is regenerated when larva is transformed into adult. Given that complete reorganization of body occurs and initiation of differentiation of imaginal primordial cells, pupal stage provides an ideal opportunity to regenerate the lost appendages. Not all organisms have the potential to regenerate. The evolutionary events that may have engendered limb regeneration has been a popular subject of speculation among researchers for long. Limb regeneration in ladybird beetles is a relatively recent phenomenon. Wang et al. (2015) reported in *Harmonia axyridis* (Pallas) that the amputated legs of larvae regenerated in adults and this occurred during pupation. The capacity of limb regeneration had been reported to be broadly conserved in coccinellid and genes responsible for regeneration are activated in pupal stage (Michaud et al., 2020). Out of 16 species of ladybird beetles, 15 were found to exhibit limb regeneration with delayed pupal duration in nine species. Regeneration neither occurred from one moult to another nor the amputated legs in adults showed regeneration (Wang et al., 2015; Saxena et al., 2016). It occurred during pupal development only. The diminishing of regenerative potential with maturation could corresponds to reduced expression of damage-responsive multiple genes needed for regeneration. For instance, the imaginal discs of *Drosophila* lose the ability to regenerate with maturation (Harris et al., 2020).

Ladybird beetles are known globally for their biological potential. During mass rearing, it is not uncommon for larvae to incur nonlethal injuries including appendage loss owing to fierce cannibalism exhibited by these beetles (Michaud 2003). The loss of limbs put constraints on the morphology of beetles and alters their biomechanics. Consequently, affecting the performance and behaviour of the injured/amputated organism (Gillis and Higham, 2016).

Thus, to survive in a competitive environment, it is required by the animal to regenerate the lost appendage.

The regeneration in *Drosophila* occurs via imaginal disc formation while in *Gryllus* regeneration involves blastema formation; the signalling involved in regenerative process is similar in both (Das. 2015). However, its yet to be explored if redevelopment of lost limbs in pupa is a part of metamorphosis or involves the process of regeneration either via blastema formation. It was hypothesized that limb regeneration in holometabolous insects like ladybird beetles, is the embryonic recapitulation of the gene programs in the pupa and is therefore independent of blastema. Therefore, this experiment will help us to understand if the ability to form a blastema is directly related to the ability to regenerate lost legs or is a part of metamorphosis.

Materials and methodology

Study Organism

Cheilomenes sexmaculata was selected for this study because it exhibits limb regeneration (Saxena et al., 2016) and is widely distributed in the local agricultural fields of India.

Stock Maintenance

Adults of *C. sexmaculata* (n=20) were collected from the fields of Lucknow, India (26°50'N, 80°54'E), and were reared for two generations in the laboratory. They were provided with an *ad libitum* supply of *Aphis nerii* (Hemiptera: Aphididae) on the host plant *Calotropis procera* (Family: Apocynaceae) commonly known as Milkweed Aphid which is found locally in abandoned lands. Stock culture of beetles was done in a 1 litre plastic beaker and kept in temperature-controlled room set to 27±2°C, 65±5% R.H. Mated females were removed from beakers and transferred to plastic Petri dishes (9.0×2.0 cm) and provisioned *ad libitum* cowpea aphids. The eggs laid were collected every 24h, and observed for hatching and the first instar larvae were used in the experiment.

Experimental Design

120 newly hatched larvae from the stock were placed in two sets of 40 each and kept singly in Petri dishes (9.0×2.0 cm). In the first set, amputation was performed in L3 stage and after 24hrs the epidermal growth at the proximal position of amputation site was scraped off. In the second set there was amputation in L3 stage but no scrapping off was performed while in third set there was no amputation and was called the control. All three sets were maintained on *ad libitum* supply of *A. nerii* (Hemiptera: Aphididae). Petri dishes were cleaned every 24 hours throughout the experiment to maintain the prey quality and to avoid any microbial contamination.

Limb amputation

The amputation was done at the third instar larva within 24 hours of moulting. At first, the larvae were chilled individually for 5 min in a refrigerator and were amputated at the proximal end of the femur of the right foreleg using. Amputation was performed under a stereomicroscope (Magnus)with the magnification 16X with the help of a micro-scalpel. After 24 hours, larvae from set one were checked under the microscope for the formation of the scab at the proximal position of the amputated site. The visible scab along with some epidermal cell-growth was scraped off with the help of a micro-scalpel. This procedure was repeated every 24 hours to scrape off any scab which formed in the foreleg to prevent the formation of blastema till the larvae reached their pre-pupal stage. The amputated larvae were reared under the above-mentioned laboratory conditions and were provisioned daily with *ad libitum* aphids, until the emergence of the adult. Larvae were examined daily to record their developmental transitions-Instar to Instar Developmental Period (DP), Pupal Duration (PD), and Post Amputation Developmental Duration (PADD), for amputated (scraped), amputated (unscraped) and control beetles.

Statistical Analysis

Data sets were first tested for normality using the Kolmogorov-Smirnov test and were found to be non-normal for PADD, L3D, L4D, PD of Control, Unregenerated and Regenerated Beetles as treatments. On finding the data non-normal, data were subjected to Generalized Linear Model (GLM) with Gamma log link. Treatments were taken as fixed factors and PADD, L3D, L4D, and PD were taken as dependent factors. This analysis was followed by a comparison of means using Wald Chi-Square test. The analysis was done using IBM SPSS version 20 statistical software.

Results and Discussion

Out of a total of 40 replicates, only 6 regenerated the amputated leg, while the rest did not regenerate (Figure 1). The everyday scrapping off of the tip of the regenerating leg corresponds to removal of blastema. Thus, our results demonstrated the importance of blastema for the regeneration of lost limbs in ladybird beetles. The 6 individuals that showed some level of regeneration of the leg amputated in the larval stage, could be the failure to completely scrape off the blastema. Thus, even some remaining cells of blastema initiated regeneration but were not enough to lead to complete development of the lost leg.

The proximal part of the amputated site, gets covered by a scab of epidermal cells. Below the scab, cellular proliferation and migration of surrounding cells occurs resulting in the formation of blastema and activation of regenerative signals. Blastema with high proliferative potency initiated regeneration of the amputated limb structure. For instance, Das (2015) reported that limb regeneration in insects is initiated via scab formation, followed by migration of epidermal cells at the site of amputation and these epidermal cells known as blastema, acted as the precursors of the regenerating cells. Various cellular processes and signalling unfold in blastema ultimately specifying the cells to give rise to the formation of lost structures (Zhong et al., 2023). There is a combination of genetic factors forming a 'prepattern', which presages the final differentiated structure. For example, several genes like *dachshund* (Dac) aid in regeneration and their absence resulted in abnormally shortened legs (Lee et al., 2013). Dac silenced in amputated larval leg, affected the patterning of the adult leg, indicating that larval appendage morphology is inherited by the adult appendages.

Amputation of foreleg led to delayed post amputation development duration with maximum delay in unscraped treatment (Table 1). Pupal duration was found to be maximum in unscraped treatment followed by scraped treatment and control. Interestingly, the pupal

duration in scraped treatment was similar to control. This difference from previous studies where maximum delay was reported in the pupal stage (Wang et al., 2015; Saxena et al., 2016; Michaud et al., 2020; Rai et al., 2023), can be attributed to the absence of blastemal cells for the initiation of regeneration and hence no regeneration occurred in pupa. While delay in the developmental duration of the fourth larval stage of the amputated treatment demonstrated the attempt to form the blastema via cellular proliferation, with no blastema in the larvae entering pupation there was no initiation of regeneration. Since, regeneration in ladybird beetles occurs during pupal duration (Wang et al., 2015; Saxena et al., 2016; Michaud et al., 2020; Zhou et al., 2021), with no regeneration taking place in the pupa, the amputated larvae of scraped off treatment showed no delay in the pupa. This explains that the delay in the pupa is indicative of regenerative processing occurring inside the pupa.

Our hypothesis that the legs amputated in the larval stages, are redeveloped during metamorphosis as a part of embryonic recapitulation of the gene programs in the pupa, was rejected. Hence, this study concludes that (i) scrapping of scab formed at the proximal position of the amputation site, prevented the formation of blastema and, (ii) the ability to form a blastema is directly related to the insects' ability to regenerate lost legs. This study concluded that limb regeneration in ladybird beetle *C. sexmaculata* is blastema dependent and the lost structure is reconstructed from blastema. Further studies are needed to understand the role of biotic and abiotic factors on blastema development, Besides, transcriptome analysis of pupa with blastema and without blastema is needed to further understand the molecular aspect of limb regeneration.

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Table 1: Results of GLM with Gamma log link depicting the effect of amputation on Post amputation development duration (PADD), larval development durations (L_3 and L_4) and pupal duration in *Cheilomenes sexmaculata*, along with their mean values.

Parameters	Wald-Chi square	P- value	df	Mean values	
PADD	304.790	0.000	2	Unscraped	(2.472±0.022) days
				Scraped	(2.206±0.022) days
				Control	(1.935±0.022) days
L_3 developmental duration	38.797	0.000	2	Unscraped	(1.021±0.059) days
				Scraped	(0.655±0.059) days
				Control	(0.516±0.059) days
L_4 developmental duration	107.745	0.000	2	Unscraped	(1.322±0.043) days
				Scraped	(1.386±0.043) days
				Control	(0.811±0.043) days
Pupal duration	399.633	0.000	2	Unscraped	(1.672±0.022) days
				Scraped	(1.147±0.022) days
				Control	(1.107±0.022) days

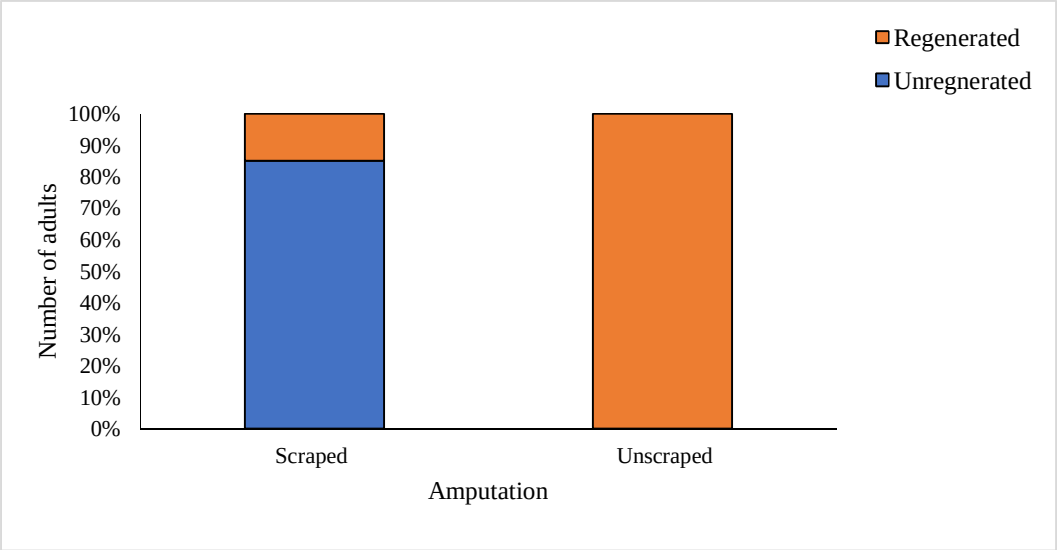

Figure 1: Effect of scraping off of proximal portion of amputation site on regeneration in comparison to the unscraped larvae of *Cheilomenes sexmaculata*.