

Limb regeneration modulates reproductive attributes in ladybirds in *Propylea dissecta* and *Coccinella septempunctata*

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In holometabolous insects, adult develops through larval and pupal stages. Limb regeneration has an impact on different life traits of organisms. In the present study, we found that limb regeneration of two ladybird species of different body size affected their life attributes. The fourth instar of *Propylea dissecta* and ladybird *Coccinella septempunctata* were taken from laboratory stock and ablated. Larvae were observed until adult emergence. Emerged adults were kept in different mating treatments. Unregenerated adults of ladybirds took more time to commence mating with shorter copulation and reduced fecundity and per cent viability. Thus, it can be concluded that regeneration ability modulates life attributes of ladybirds, irrespective of body size.

Keywords: Body size, fecundity, ladybirds, limb regeneration, reproductive attributes.

REGENERATION is beneficial to insects in terms of physical fitness. It also modulates life attributes. Males have been known to regulate the courtship, copulation duration, quality and quantity of ejaculates depending upon the surrounding environment and their physical fitness¹⁻³. Poorly regenerated or physically disabled males are known to affect mobility, mating success and reproduction⁴. Besides, it has been reported that the missing or regenerated limbs are known to affect the outcome of various ecological interactions, such as prey-predator interactions and intraspecific competition^{5,6}. In wolf spider *Schizocosa ocreata*, it has been reported that the prey capturing efficiency decreased due to a missing or regenerating limb. In field cricket *Gryllus bimaculatus*, it has been reported that in females, loss of limb has resulted in significantly reduced mating ability, while in males it has resulted in reduced longevity. Under promiscuous conditions, poor quality males were overpowered by healthy males^{7,8}.

In terms of reproductive performance in spiders, it has been reported that loss of forelegs can lead to reduced reproductive success by fewer sperm transfers⁹. Studies on *Harmonia axyridis* showed that the unregenerated adults were poor in mating and reproductive parameters than the regenerated and non-ablated individuals¹⁰. Ladybirds are polyandrous^{11,12} and display mate choice^{13,14}. In *Menochilus sexmaculatus* Fabricius, it has been shown that regenera-

tion occurs in the adult stage and not from instar to instar, and unregenerated adults were poor performers in mating and reproductive parameters¹⁵. Another study on *M. sexmaculatus* reported that when the adult legs were ablated from three different joints, there was a difference in their mating performance and reproductive output¹⁶.

Here, we aim to study the effects of limb regeneration on two ladybird species, i.e. *Coccinella septempunctata* (L.) and *Propylea dissecta* (Mulsant). In this study, we have hypothesized that regeneration may have some costs in terms of reproductive attributes. This was examined using fourth instar individuals of *P. dissecta* and *C. septempunctata* that were amputated for their forelimb from the base of the coxa. Regenerated, normal and unregenerated adults of the two ladybird species were used in different mating treatments, and the impact on mating and reproductive attributes of both beetles was recorded.

Live adults of medium-sized *P. dissecta* (average fresh wt: 13.04 ± 0.15 mg, *n* = 50) and large sized *C. septempunctata* (average fresh wt: 21.70 ± 0.15 mg, *n* = 50) were collected from the agricultural fields of Lucknow, Uttar Pradesh, India (26°50'N, 80°54'E). These beetles were selected for experimentation due to their predominance in local fields, wide prey range¹⁷ and high reproductive output. Males and females were paired in plastic petri dishes (9.0 × 2.0 cm) and provided with an ad libitum supply of cowpea aphid *Aphis craccivora* Koch (Hemiptera: Aphididae) raised on cowpea *Vigna unguiculata* L. in a glasshouse at 25° ± 2°C, 65 ± 5% relative humidity (RH). Petri dishes with mating pairs were placed in BOD incubators (Yorco Super Deluxe, YSI-440, New Delhi) at 27° ± 1°C, 65 ± 5% RH, 14L : 10D. They were inspected twice daily (1000 and 1500 h) for oviposition. The eggs were separated and reared individually in petri dishes until the emergence of the fourth instar individuals.

Fourth instar larvae (24-h-old post-moulting) of *P. dissecta* and *C. septempunctata* were divided into two groups of 100 individuals each. One group was reared as control (termed as normal hereafter) and chilled for 5 min with no amputation treatment. The other group was also chilled for 5 min to ease the ablation process. Amputation of forelimb from base of coxa of right side was done under a stereoscopic binocular microscope (Magnus) at 16× magnification with the help of a micro-scalpel. Post-amputation, larvae were reared individually in petri dishes until adult emergence. All the adults were isolated and reared on the ad libitum supply of *A. craccivora*.

Ten-day-old unmated adults of both species were selected for the amputation treatment and assessed for limb regeneration. The ones with limb regeneration were called regenerated (in *P. dissecta* the limbs were incompletely regenerated, so here we used the term 'incompletely regenerated adults') and those without regeneration were called unregenerated. These and the normal adults were allowed to mate in the following combinations (1) regenerated♂ × regenerated♀ (♂_R × ♀_R), (2) regenerated♂ × normal♀ (♂_R × ♀_N),

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(3) regenerated ♂ × unregenerated ♀ ($\sigma_R \times \varphi_U$), (4) unregenerated ♂ × unregenerated ♀ ($\sigma_U \times \varphi_U$), (5) unregenerated ♂ × normal ♀ ($\sigma_U \times \varphi_N$), (6) unregenerated ♂ × regenerated ♀ ($\sigma_U \times \varphi_R$), (7) normal ♂ × normal ♀ ($\sigma_N \times \varphi_N$), (8) normal ♂ × unregenerated ♀ ($\sigma_N \times \varphi_U$), and (9) normal ♂ × regenerated ♀ ($\sigma_N \times \varphi_R$). In the case of *P. dissecta*, complete regeneration was not observed. Therefore, incompletely regenerated adults were used for the above mating combinations. Pairs were allowed to mate until they disengaged naturally. Time to commence mating (TCM; introduction in petri dish to establishment of genital contact) and copulation duration (CD; from the establishment of genital contact till natural disengagement) were recorded. Females were separated and kept individually in petri dishes with an ad libitum supply of *A. craccivora*. They were inspected twice (10:00 and 15:00 h) daily for oviposition for the next seven days and the egg hatching was recorded. All the mating combinations were replicated 15 times.

Data on mating (time of commencement of mating and copulation duration) and reproductive attributes (fecundity and per cent egg viability (dependent factors)) were initially tested for normal distribution (Kolmogorov–Smirnov test). On being found normally distributed with homogeneous variation, data on mating and reproductive attributes were subjected to a two-way analysis of variance (ANOVA) with regeneration status of male and female as independent factors. This analysis was followed by a comparison of means using post-hoc Tukey's honest significance test at 5%. All statistical analyses were done using R studio version 1.2.1335.

In *P. dissecta*, regeneration status of males and females was found to have significant effect on TCM ($F_{\sigma} = 7.05$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 27.88$, $P < 0.05$, $df = 2,126$). The interaction between the two independent factors was insignificant ($F_{\sigma \times \varphi} = 1.89$, $P > 0.05$, $df = 4,126$). A significant effect of regeneration status of males and females was also observed on TCM of mating in *C. septempunctata* ($F_{\sigma} = 48.43$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 65.51$, $P < 0.05$, $df = 2,126$). The interaction between the two factors was also found significant ($F_{\sigma \times \varphi} = 7.69$, $P < 0.05$, $df = 4,126$). The highest TCM was found in *P. dissecta* and *C. septempunctata* when unregenerated males were allowed to mate with unregenerated females (Tables 1 and 2). However, in the case of *P. dissecta*, lowest TCM was observed when normal males mated with normal females, which was contrary to the case of *C. septempunctata* where the lowest TCM was observed when regenerated adults were allowed to mate (Table 1).

CD was also found to have a significant effect of regeneration status of males and females in *P. dissecta* ($F_{\sigma} = 175.55$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 195.35$, $P < 0.05$, $df = 2,126$) as well as in *C. septempunctata* ($F_{\sigma} = 343.90$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 333.08$, $P < 0.05$, $df = 2,126$). The interactions between males and females were also found significant in *P. dissecta* ($F_{\sigma \times \varphi} = 36.12$, $P < 0.05$, $df = 4,126$) and *C. septempunctata* ($F_{\sigma \times \varphi} = 74.36$, $P < 0.05$,

$df = 4,126$). The shortest CD was recorded in unregenerated treatments. In *P. dissecta*, the longest CD was found when normal males were paired with normal females, while in *C. septempunctata*, maximum CD was recorded when regenerated adults were paired (Tables 1 and 2).

Fecundity was significantly influenced by the regeneration status of males and females in *P. dissecta* ($F_{\sigma} = 1324.81$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 42.79$, $P < 0.05$, $df = 2,126$) and *C. septempunctata* ($F_{\sigma} = 763.5$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 1018.7$, $P < 0.05$, $df = 2,126$). The interactions were also significant in both *P. dissecta* ($F_{\sigma \times \varphi} = 4.98$, $P < 0.05$, $df = 4,126$) and *C. septempunctata* ($F_{\sigma \times \varphi} = 299.2$, $P < 0.05$, $df = 4,126$). In *P. dissecta* maximum fecundity was recorded in normal adults, while in *C. septempunctata* it was found in regenerated adults (Tables 1 and 2). In both the ladybirds, minimum fecundity was observed for the unregenerated pairs (Tables 1 and 2). A significant effect of regeneration status of males and females was also recorded for the per cent egg viability of *P. dissecta* ($F_{\sigma} = 1051.78$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 10.42$, $P < 0.05$, $df = 2,126$) and *C. septempunctata* ($F_{\sigma} = 126.32$, $P < 0.05$, $df = 2,126$, $F_{\varphi} = 22.07$, $P < 0.05$, $df = 2,126$). The interactions of these factors were also significant in *P. dissecta* ($F_{\sigma \times \varphi} = 8.72$, $P < 0.05$, $df = 4,126$), but insignificant in *C. septempunctata* ($F_{\sigma \times \varphi} = 2.02$, $P > 0.05$, $df = 4,126$). Minimum per cent egg viability was recorded for the unregenerated treatments in both the ladybird beetles, while maximum egg viability was found for the normal treatments in *P. dissecta* and the regenerated treatments for *C. septempunctata* (Tables 1 and 2).

In the present study, we recorded the longest TCM and shortest CD for the unregenerated treatments in both ladybirds. Similarly, fecundity and per cent egg viability were minimum in the unregenerated treatments for both the ladybird species.

It was found that the unregenerated and incompletely regenerated adults in *P. dissecta* took more time to commence mating and mated for shorter duration compared to the regenerated and normal adults. This can probably be attributed to the rejection behaviour of the females post-assessment of the physical condition of males^{18–20}. Another reason for the lower performance of unregenerated and incomplete adults could be incomplete physical contact with their mates due to the missing limb¹⁶. This supports the hypothesis of the honest display of signals^{21–24}, where individuals with better physical fitness get more chances for mating. Studies on insects and higher animals have revealed that male ornamentation is inversely proportional to its fighting and mating success^{25–28}. The reduced fecundity and per cent egg viability by the unregenerated and incomplete regenerated adults may be attributed to (a) the lower mating duration as reported earlier²⁹ that longer copulation duration results in increased paternity share due to a larger number of spermatophore transfer, and (b) utilization of sperms by females owing to the perception of reduced fitness of males^{24,30}. Differential usage of sperms by females

Table 1. Mating and reproductive parameters of *Coccinella septempunctata* when adults were kept under different mating trials

Male type	Female type	Time of commencement of mating (min)	Copulation duration (min)	Fecundity	Egg viability (%)
Regenerated	Regenerated	5.733 ± 0.44 ^a	126.46 ± 2.72 ^c	129.26 ± 1.28 ^c	95.86 ± 0.17 ^c
	Normal	9.06 ± 0.74 ^b	117.13 ± 1.76 ^b	110.4 ± 0.97 ^b	92.45 ± 0.17 ^b
	Unregenerated	14.53 ± 0.78 ^c	49.06 ± 4.74 ^a	86.6 ± 0.70 ^a	88.45 ± 0.27 ^a
Normal	Regenerated	5.73 ± 0.44 ^a	126.26 ± 2.01 ^c	123.73 ± 0.73 ^b	94.93 ± 0.23 ^c
	Normal	8.06 ± 0.72 ^b	124.2 ± 2.62 ^b	122.06 ± 1.01 ^b	93.17 ± 0.32 ^b
	Unregenerated	15 ± 0.69 ^c	47.66 ± 2.02 ^a	82.6 ± 0.48 ^a	82.74 ± 0.45 ^a
Unregenerated	Regenerated	14.4 ± 0.84 ^b	47.26 ± 2.39 ^b	87.06 ± 0.49 ^a	75.51 ± 0.53 ^a
	Normal	13.8 ± 0.65 ^a	47.66 ± 2.92 ^b	87.53 ± 0.48 ^a	75.03 ± 0.51 ^a
	Unregenerated	16.4 ± 1.12 ^c	44.4 ± 2.90 ^a	87.6 ± 0.42 ^a	70.79 ± 4.57 ^a

Values are mean ± SE. Small letters represent the comparison of means between the mating and reproductive parameters of different mating trials. Similar letters indicate lack of significant difference at $P > 0.05$.

Table 2. Mating and reproductive parameters of *Propylea dissecta* when adults were kept under different mating trials

Male type	Female type	Time of commencement of mating (min)	Copulation duration (min)	Fecundity	Egg viability (%)
Incomplete regenerated	Incomplete regenerated	13.2 ± 0.67 ^b	96.6 ± 3.09 ^b	44.46 ± 0.43 ^b	82.62 ± 0.44 ^a
	Normal	11.2 ± 0.64 ^a	100.13 ± 1.55 ^c	48 ± 0.56 ^c	82.39 ± 0.55 ^a
	Unregenerated	16.06 ± 0.91 ^c	45.13 ± 2.36 ^a	40.6 ± 0.54 ^a	82.23 ± 0.35 ^a
Normal	Incomplete regenerated	12.33 ± 0.68 ^b	69 ± 3.87 ^b	62.26 ± 0.44 ^b	90.05 ± 0.41 ^b
	Normal	8.46 ± 0.88 ^a	109.6 ± 1.98 ^c	64.73 ± 0.91 ^c	92.43 ± 0.25 ^c
	Unregenerated	16.06 ± 0.91 ^c	47 ± 1.99 ^a	61.6 ± 0.67 ^a	87.86 ± 0.44 ^a
Unregenerated	Incomplete regenerated	14.4 ± 0.84 ^b	39.86 ± 2.07 ^b	38 ± 0.71 ^b	73.96 ± 0.40 ^a
	Normal	13.6 ± 0.76 ^a	50.13 ± 4.55 ^c	37.86 ± 0.67 ^b	73.96 ± 0.40 ^a
	Unregenerated	16.66 ± 1.17 ^c	35.66 ± 1.43 ^a	33.93 ± 0.66 ^a	73.82 ± 0.54 ^a

Values are mean ± SE. Small letters represent the comparison of means between the mating and reproductive parameters of different mating trials. Similar letters indicate lack of significant difference at $P > 0.05$.

owing to the status of males has been well established^{20,31}. Males with lower viability may also be attributed to the wrong positioning of the aedeagus due to a lack of physical fitness. Shandilya *et al.*¹⁶ showed that the reduced viability in leg-impaired *M. sexmaculatus* can be due to the lack of structures which are required for proper holding of mates. No positive relation between the CD and the reproductive attributes was recorded in both ladybirds for different mating treatments. This suggests a role for assessment of the physical fitness of males and females that may lead to the differential investment in mating and thus differences in fecundity and per cent egg viability.

In conclusion, the present study revealed that regeneration in both the ladybird species modulates the mating and reproductive attributes, and unregenerated adults perform poorly, irrespective of the body size of the ladybirds.

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Successful separation and detection of gold sulphide in ore samples from the banded iron formation of Goa, India

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Gold (I) sulphide (Au₂S) is an inorganic compound and the principal sulphide of gold. This study aimed to separate gold sulphide from ore samples such as banded hematite quartzite mined from the banded iron formation of Goa, India. The samples were dried, powdered and then concentrated by the panning method. The coarse ferromagnetic material was separated and sieved into different fractions of varying sieve sizes, i.e. 250, 150, 106 and 53 µm. Next, they were subjected to serial washing, and after drying, were tested for the presence of gold by ICP-AES, optical microscopy, phase contrast microscopy, SEM-EDS and total content of the sulphur was analysed by CHNS/O elemental analyzer.

Keywords: Banded iron formation, detection, gold sulphides, ore samples, separation.

GOA forms a part of the northwestern Western Dharwar Craton (WDC), which serves Asia's major metallogenic province¹. The iron ores of Goa are associated with greenstone and occur as banded hematite quartzite (BHQ) and banded magnetite quartzite (BMQ)². Previous research findings show the presence of gold in the WDC and parts of the Konkan region, India. Reports are available on the occurrence of gold (0.06–0.16 ppm) in lateritic powdery ore of Keri and Kalne villages and <0.1 ppm gold in laterite and iron ores in Sindhudurg district, Maharashtra, India³. Studies have established that BHQ in Goa, India, contains 12–13 ppm of gold⁴. The occurrence of gold in sulphidic banded iron formations (BIFs) ranged from 0.7 to 3.2 ppm (ref. 5). Gold(I) sulphide having the formula Au₂S is an inorganic compound and principal sulphide of gold. Gold usually occurs in its native form in the lattice of sulphides (especially arsenopyrite, pyrite and chalcopyrite), carbonate, silicate and oxide minerals. For sustainable development of the gold industry, recovery of gold from refractory sulphide gold ores is important. However, there are many challenges in the treatment of sulphide refractory gold ores. The incorporation of gold into sulphide minerals has long been inferred by several investigators⁶. In this study, a novel method for gold sulphide recovery from iron ore samples in the BIF of Goa has been successfully used with prospects of bio-mining of gold as a long-term objective.

A significant part of the primary gold reserves of the world is contained in sulphide ores, many types of which

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