

1 **Limb regeneration modulates reproductive attributes in ladybirds: A case study in**

2 *Propylea dissecta* and *Coccinella septempunctata*

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15 **Short running title:** Limb regeneration in ladybirds

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25 **Abstract**

26 In holometabolous insects, adult develops through larval and pupal stages. Limb regeneration has
27 impact on different life traits of organisms. In present study, we investigated limb regeneration of
28 two different sized ladybirds affect life attributes. Fourth instar of *Propylea dissecta* and ladybird
29 *Coccinella septempunctata* were taken from laboratory stock and were ablated. Larvae were
30 observed until adult emergence. Emerged adults were kept in different mating treatments.
31 Unregenerated adults of ladybirds took more time to commence mating with shorter copulation
32 and reduced fecundity and percent viability. It is concluded that regeneration ability modulates
33 life attributes of ladybirds irrespective of body size.

34

35 **Keywords:** Coccinellidae, Coleoptera, forelimb, fecundity, regenerated, unregenerated

36 Regeneration is beneficial to insects in terms of physical fitness. It also modulates the life
37 attributes. Males have been known to regulate the courtship, copulation duration, quality and
38 quantity of ejaculates depending upon the surrounding environment and their physical fitness^{1,2,3}.
39 Poorly regenerated or physically disabled males were known to affect the mobility, mating
40 success and reproduction⁴. Besides this, it has been reported that the missing or regenerated limb
41 are known to affect the outcome of the various ecological interactions such as prey-predator

42 interactions and intraspecific competition^{5,6}. In wolf spider, *Schizocosa ocreata* it has been
43 reported that their prey capturing efficiency decreased due to missing or regenerating limb. In
44 field cricket *Gryllus bimaculatus* it has been reported that in females, loss of limb has resulted in
45 significantly reduced mating ability while in males it has resulted in reduced longevity. Under
46 promiscuous conditions, poor quality males were overpowered by the healthy males^{7,8}.

47 In terms of reproductive performance, in spiders, it has been also reported that loss of
48 forelegs can lead to the reduced reproductive success by less transfer of sperms⁹. Studies in
49 *Harmonia axyridis* have also reported that the unregenerated adults were poor performers in
50 terms of mating and reproductive parameters than the regenerated and non-ablated individuals¹⁰.
51 Ladybirds are polyandrous^{11,12} and display mate choice^{13,14}. In *Menochilus sexmaculatus*
52 Fabricius it has been shown earlier that the regeneration occurs in adult stage and not from instar
53 to instar and unregenerated adults were poor performers in terms of mating and reproductive
54 parameters¹⁵. Another study on *M. sexmaculatus* has reported when the adult legs were ablated
55 from three different joints then there was the difference in their mating performance and the
56 reproductive output¹⁶.

57 In the present study, we aim to study the effects of limb regeneration on two ladybirds *i.e.*
58 *Coccinella septempunctata* (L.) and *Propylea dissecta* (Mulsant). In this study we have
59 hypothesised that regeneration may have some costs in terms of reproductive attributes. This was

60 examined using fourth instar individuals of *P. dissecta* and *C. septempunctata* that were
61 amputated for their forelimb from the base of coxa. Regenerated, normal and unregenerated
62 adults of the two ladybird species were used in different mating treatments and its impact on
63 mating and reproductive attributes of both the beetles were recorded.

64 *Stock maintenance*

65 Live adults of medium sized *Propylea dissecta* (average fresh weight: 13.04±0.15 mg, n = 50)
66 and large sized, *Coccinella septempunctata* (average fresh weight: 21.70±0.15 mg, n=50) were
67 collected from the agricultural fields of Lucknow, India (26°50'N, 80°54'E). These beetles were
68 selected for experimentation due to their predominance in local fields, wide prey range¹⁷ and
69 high reproductive output. Males and females were paired in plastic Petri dishes (hereafter,
70 9.0×2.0 cm) and provided with *ad libitum* supply of cowpea aphid, *Aphis craccivora* Koch
71 (Hemiptera: Aphididae) raised on cowpea, *Vigna unguiculata* L. in a glasshouse at 25 ±2°C, 65
72 ±5% Relative Humidity. Petri dishes with mating pairs were placed in BOD incubators (Yorco
73 Super Deluxe, YSI-440, New Delhi, India) at 27 ± 1°C, 65 ±5% R.H., 14L:10D. Those were
74 inspected twice daily (1000 and 1500 h) for oviposition. The eggs were separated and reared
75 individually in Petri dishes until the emergence of fourth instar individuals.

76 *Limb amputation*

77 Fourth instar larvae (24h old post-moulting) of *P. dissecta* and *C. septempunctata*, were divided
78 into two groups of 100 individuals each. One group was reared as control (termed as normal

79 hereafter) and chilled for 5 minutes with no amputation treatment. The other group of larvae
80 were also chilled for 5 minutes to ease the ablation process. Amputation of fore limb from base
81 of coxa of right side was done under a stereoscopic binocular microscope (Magnus) at 16x
82 magnification with the help of micro-scalpel. Post amputation, larvae were reared individually in
83 Petri dishes until adult emergence. All the adults were isolated and reared on the *ad libitum*
84 supply of *A. craccivora*.

85 ***Mating combinations and attributes***

86 10-day old unmated adults of both the species were taken from the amputation treatment and
87 were assessed for limb regeneration. The ones with limb regeneration were called regenerated (in
88 *P. dissecta*, limbs were incompletely regenerated so here we used incompletely regenerated
89 adults) and those without regeneration were called unregenerated. These adults and normal
90 adults were allowed to mate in following combinations (1) Regenerated♂ × Regenerated♀ (♂_R ×
91 ♀_R), (2) Regenerated♂ × Normal♀ (♂_R × ♀_N), (3) Regenerated♂ × Unregenerated♀ (♂_R × ♀_U),
92 (4) Unregenerated♂ × Unregenerated♀ (♂_U × ♀_U), (5) Unregenerated♂ × Normal♀ (♂_U × ♀_N), (6)
93 Unregenerated♂ × Regenerated♀ (♂_U × ♀_R), (7) Normal♂ × Normal♀ (♂_N × ♀_N), (8) Normal♂ ×
94 Unregenerated♀ (♂_N × ♀_U), and (9) Normal♂ × Regenerated♀ (♂_N × ♀_R). In case of *P. dissecta*,
95 complete regeneration was not observed. Therefore, incompletely regenerated adults were used
96 for forming above mating combinations. Pairs were allowed to mate until they disengaged
97 naturally. Time to commence mating (introduction in Petri dish to establishment of genital
98 contact) and copulation duration (from establishment of genital contact till natural
99 disengagement) were recorded. Females were separated and kept individually in Petri dishes
100 with *ad libitum* supply of *A. craccivora*. They were inspected twice (10:00 and 15:00hr) daily for

101 oviposition for next seven days and the egg hatching was recorded. All the mating combinations
102 were replicated 15 times.

103 *Statistical Analysis*

104 Data on mating (time of commencement of mating and copulation duration) and reproductive
105 attributes (fecundity and percent egg viability (dependent factors) were initially tested for normal
106 distribution (Kolmogorov-Smirnoff test). On being found normally distributed with
107 homogeneous variation, data on mating and reproductive attributes were subjected to two-way
108 analysis of variance (ANOVA) with regeneration status of male and female as independent
109 factors. This analysis was followed by comparison of means using post hoc Tukey's honest
110 significance test at 5%. All statistical analyses were conducted using R studio Version 1.2.1335
111 statistical software.

112 In *P. dissecta*, regeneration status of males and females were found to have significant effect on
113 the time to commence mating (TCM) ($F_{\delta}=7.05$, $P<0.05$, $df=2,126$; $F_{\varphi}=27.88$, $P<0.05$, $df=2$,
114 126). The interaction between the two independent factors was insignificant ($F_{\delta\times\varphi}=1.89$; $P>0.05$;
115 $df=4,126$). A significant effect of regeneration status of male and female was also observed on
116 time of commencement of mating in *C. septempunctata* ($F_{\delta}=48.43$; $P<0.05$; $df=2,126$; $F_{\varphi}=65.51$;
117 $P<0.05$; $df=2, 126$). The interaction between the two factors was also found significant
118 ($F_{\delta\times\varphi}=7.69$; $P<0.05$; $df=4,126$). The highest time to commence mating was found in *P. dissecta*
119 and *C. septempunctata* when unregenerated males were allowed to mate with unregenerated
120 females (Table 1 and 2). However, in case of *P. dissecta*, lowest time to commence mating was
121 observed when normal males mated with normal females, which was contrary to *C.*

122 *septempunctata* where lowest TCM was observed when regenerated adults were allowed to mate
123 (Table 1).

124 Copulation duration (CD) was also found to have a significant effect of regeneration status of
125 males and females in *P. dissecta* ($F_{\delta}=175.55$; $P<0.05$; $df=2,126$; $F_{\varphi}=195.35$; $P<0.05$; $df=2, 126$)
126 as well as in *C. septempunctata* ($F_{\delta}=343.90$; $P<0.05$; $df=2,126$; $F_{\varphi}=333.08$; $P<0.05$; $df=2, 126$).
127 The interactions between status of males and females were also found significant in *P. dissecta*
128 ($F_{\delta\times\varphi}=36.12$; $P<0.05$; $df=4,126$) and *C. septempunctata* ($F_{\delta\times\varphi}=74.36$; $P<0.05$; $df=4,126$). The
129 shortest copulation duration was recorded in unregenerated treatments. In *P. dissecta*, longest
130 copulation duration was found when normal males were paired with normal females, while in *C.*
131 *septempunctata* the maximum copulation duration was recorded when regenerated adults were
132 paired (Table 1 and 2).

133 *Reproductive attributes*

134 Fecundity was significantly influenced by the regeneration status of males and females in *P.*
135 *dissecta* ($F_{\delta}=1324.81$; $P<0.05$; $df=2,126$; $F_{\varphi}=42.79$; $P<0.05$; $df=2, 126$) and *C. septempunctata*
136 ($F_{\delta}=763.5$; $P<0.05$; $df=2,126$; $F_{\varphi}=1018.7$; $P<0.05$; $df=2, 126$). The interactions were also
137 significant in both *P. dissecta* ($F_{\delta\times\varphi}=4.98$; $P<0.05$; $df=4,126$) and *C. septempunctata*
138 ($F_{\delta\times\varphi}=299.2$; $P<0.05$; $df=4,126$). In *P. dissecta*, maximum fecundity was recorded in normal
139 adults while in *C. septempunctata* it was found in regenerated adults (Table 1 and 2). In both the

140 ladybirds minimum fecundity was for unregenerated pairs (Table 1 and 2). A significant effect
141 of regeneration status of males and females was also recorded for the percent egg viability of
142 *P.dissecta* ($F_{\delta}=1051.78$; $P<0.05$; $df=2,126$; $F_{\varphi}=10.42$; $P<0.05$; $df=2, 1326$) and
143 *C.septempunctata*($F_{\delta}=126.32$; $P<0.05$; $df=2,126$; $F_{\varphi}=22.07$; $P<0.05$; $df=2, 126$). The interaction
144 of these factors were also significant in *P. dissecta* ($F_{\delta\times\varphi}=8.72$; $P<0.05$; $df=4,126$) but
145 insignificant in *C. septempunctata* ($F_{\delta\times\varphi}=2.02$; $P>0.05$; $df=4,126$). Minimum percent egg
146 viability was recorded for unregenerated treatments in both the ladybird beetles while maximum
147 egg viability was found for normal treatments in *P.dissecta* and in regenerated treatments for *C.*
148 *septempunctata* (Table 1 and 2).

149 In the present study we found that longest time of commencement of mating (TCM) and shortest
150 copulation duration were recorded for unregenerated treatments in both the ladybirds. Similarly,
151 fecundity and percent egg viability were minimum in unregenerated treatments for both the
152 ladybird species.

153 It was found that the unregenerated and incompletely regenerated adults (in *P. dissecta*) adults
154 took more time to commence mating and mated for shorter duration as compared to regenerated
155 and normal adults. This can probably be attributed to the rejection behaviour of female post
156 assessment of the physical condition of males^{18,19,20}. Another reason which could be explained
157 for the lower performance of unregenerated and incomplete adults was incomplete physical

158 contact with their mates due to the missing limb¹⁶. This supports the hypothesis of the honest
159 display of signals^{21, 22, 23, 24} where the individuals with more physical fitness get more chances for
160 mating. Studies in insects and higher animals have revealed that the male ornamentation is
161 inversely proportional to their fighting success and mating success^{25,26,27,28}. The reduced
162 fecundity and percent egg viability by unregenerated and incomplete regenerated adults may be
163 attributed to (a) the lower mating duration as reported by²⁹ reported that longer copulation
164 durations result in increased paternity share due to larger number of spermatophore transfer and
165 (b) utilization of sperms by females owing to the perception of reduced fitness of males^{24, 30}.
166 Differential usage of sperms by females owing to the status of the males has been well
167 established^{31, 20}. Males with lower viability may also be attributed to the wrong positioning of
168 the aedeagus due to lack of physical fitness. Study by Shandilya et al. (2018) revealed the
169 reduced viability in leg impaired *M. sexmaculatus* can be due to the lack of structures which are
170 required for proper holding of mates. No positive relation between the copulation durations and
171 reproductive attributes were recorded in both the ladybirds in different mating treatments. This
172 suggests a role of assessment of the physical fitness by males and females that may lead to the
173 differential investment in mating leading to differences in fecundity and percent egg viability.
174 Thus, this study has revealed that regeneration in both the ladybirds modulates the mating and
175 reproductive attributes, and unregenerated adults perform poorly irrespective of the size of
176 ladybirds.

177 **Acknowledgements**

178 SS acknowledges Basic Science Research Fellowship by University Grants Commission, New
179 Delhi, India (F.No.25-1/2014-15 (BSR)/7-109/2007/BSR) dated August 25, 2015.

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