Bulletin of Entomological Research, Page 1 of 8
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doi:10.1017/S0007485315000036

Sexual selection drives the evolution of limb regeneration in *Harmonia axyridis* (Coleoptera: Coccinellidae)

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Abstract

When *Harmonia axyridis* larvae were subjected to amputation of a foreleg in the fourth instar, 83% survived and, of these, 75% regenerated the leg during pupation. Regenerators pupated at heavier weights than controls (unoperated) or non-regenerators, and spent longer in pupation. Regenerated males were preferred by females in choice tests and produced more viable progeny than control males. Unregenerated males were less preferred by females, copulated for shorter periods than control males, and reduced female fecundity. Amputation diminished beneficial paternal effects, whether males regenerated or not, resulting in progeny with slower development and smaller adult body mass relative to control paternity. Progeny of unregenerated males had lower survival and body mass, whether male or female, confirming that regeneration was an honest signal of mate quality. When offspring had a foreleg amputated, a regenerated paternity yielded higher survival than control paternity, but similar rates of regeneration, whereas an unregenerated paternity yielded lower rates of survival and leg regeneration than control paternity. Regenerating beetles were twice as likely to be melanic as non-regenerating or control beetles, suggesting pleiotropic effects of melanism on processes involved in regeneration. This is the first report of complete limb regeneration by a holometabolous insect in the pupal stage, and the first example of sexual selection for regenerative capacity.

Keywords: body size, development, female choice, fitness, melanism, paternal effects

(Accepted 22 December 2014)

Introduction

The ability to regenerate amputated appendages has been documented in many taxa, including annelids (Yanez-Rivera & Mendez, 2014), cnidarians (Passamaneck & Martindale, 2012), planarians (Lobo *et al.*, 2012) crustaceans (Shock *et al.*, 2009), and insects (Wolpert, 2015). Among vertebrates, limb regeneration is known for some species of fish (Murciano *et al.*, 2002; Cuervo *et al.*, 2012) and lizards (Alibardi, 2012), but the capacity is most highly developed in urodele amphibians (Brockes & Gates, 2014). Salamanders, especially *Ambystoma mexicanum* (Shaw), have often been used as a model system (e.g., Nacu & Tanaka, 2011) and have revealed a complex interplay between neural (e.g., Satoh *et al.*, 2008, 2012) and endocrinological (Kumar *et al.*, 2010) factors in the process of limb regeneration from epithelial tissues.

There has been little work on limb regeneration in insects and most has focused on elucidating mechanisms of regeneration during nymphal molts in species with incomplete metamorphosis, such as cockroaches (Truby, 1983; Tanaka *et al.*, 1987), mantids (Karuppanan, 1998), bugs (Knobloch & Steel, 1988), and crickets (Lakes & Mucke, 1989; Li *et al.*, 2007). Regeneration in arthropods can occur only during the process

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of molting when the epidermis separates from the cuticle; molting is often delayed following amputation and more than one molt may be required for complete regeneration in both crustaceans (e.g., Selin, 2003) and insects (Wigglesworth, 1965). Although the pupal stage of holometabolous insects effects a transformation of the entire body plan, an ideal opportunity for regeneration, research has rarely addressed the possibility of pupal stage regeneration of limbs lost in larval stages. Singh *et al*. (2007) demonstrated that the adult legs of *Bombyx mori* (L.) (Lepidoptera: Bombycidae) develop from their larval prototypes, rather than imaginal discs, and do not regenerate either segments or complete legs ablated in the larval stage. Lee *et al*. (2013) used RNA interference techniques to demonstrate a similar continuity between larval and adult leg development processes in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Mitten *et al*. (2012) explored the proteases involved in the healing process prior to blastema formation and leg regeneration during larval molts in *T. castaneum*, but again, leg regeneration in the pupal stage was not observed (Shah *et al*., 2011).

Harmonia axyridis Pallas (Coleoptera: Coccinellidae) is a polyphagous insect predator that has often been employed in biological control, but is now a cosmopolitan invasive species causing negative ecological impacts throughout much of the world (Michaud, 2002; Koch, 2003; Roy & Wajnberg, 2008). Preliminary experiments with an *H. axyridis* population native to Beijing, China, revealed that a large proportion of larvae subjected to foreleg amputation early in the fourth instar were able to regenerate the leg during the pupal stage. Both male and female *H. axyridis* are sensitive to indicators of relative fitness in their mates, such as elytral background color which can be either red/orange (succinic) or black (melanic), the two phenotypes being alternatively advantaged or disadvantaged depending on whether they occur in the spring or fall generation (Wang *et al*., 2009). Both sexes can modulate their reproductive investment according to whether the phenotype of their mate is seasonally favored, as measured by strength of mate preference (females), duration of copula (males), and egg retention period post-copula (females). We hypothesized that regenerative ability could potentially serve as an 'honest' signal of mate quality, *sensu* the handicap principle (Zahavi, 1975; Grafen, 1990*a*, *b*; Zahavi & Zahavi, 1997), since only individuals with high fitness would be expected to survive, and repair, such a significant physiological trauma.

In the present study, we used laboratory experiments to test the consequences of leg regeneration for male fitness, female choice, and offspring fitness in *H. axyridis*. We hypothesized that (1) physiological costs of amputation would be manifest in negative impacts on pupal development, adult body size, and/or adult reproductive performance; (2) regenerative capacity would be correlated with other traits contributing to elevated fitness and; (3) females should mate preferentially with regenerators, provided they are able to distinguish them, and discriminate against non-regenerators. In addition, since males of many coccinellid species, including *H. axyridis*, use paternal effects to modulate the developmental phenotype of their progeny in beneficial ways (e.g., Michaud *et al*., 2013; Mirhosseini *et al*., 2014), we argued (4) that amputation would negatively impact offspring phenotypes via diminished paternal effects and, (5) that these impacts would be greater for progeny of non-regenerators than regenerators, assuming regenerative capacity was a property of high-fitness genotypes. Finally, we assessed the heritability of regeneration by subjecting subsets of the F1 generation to limb amputation and comparing their survival and regenerative capacity among paternal types.

Materials and methods

Insect colony

Adult *H. axyridis* (ca. 300) were collected by sweep net from a wheat field on the campus of Beijing Academy of Agriculture and Forestry Sciences (BAAFS) in Beijing, PRC. The beetles were held in aluminum frame cages (40.0 × 40.0 × 45.0 cm) covered with nylon mesh, 20 pairs per cage, and provisioned daily with *ad libitum Myzus persicae* Sulzer grown on tobacco seedlings in a climate-controlled growth chamber. The cages were held in climate-controlled growth chambers (Sanyo, MH351, Osaka, Japan) set to a constant temperature of 25.0 ± 1.0°C, RH = 60–70% and a 16:8 L:D photoperiod under a light intensity of 600 lux. All experimental insects were maintained under the same conditions as the stock colony. Eggs were collected daily and held in plastic Petri dishes (9.0 cm diam) until eclosion. Neonate larvae were reared in similar Petri dishes, five per dish, with *ad libitum M. persicae* nymphs refreshed daily until larvae pupated.

Limb amputation

A cohort of 300 *H. axyridis* larvae were reared from the offspring of five females. As larvae molted to the fourth instar, a series of 40 were selected from each maternal lineage on the basis of similarity in size, for a total of 200. From each sibling group, 30 were assigned to treatment (limb amputation) and 10 to controls. For limb amputation, newly-molted, fourth instar larvae (ca. 24 h post-molt) were chilled for 5 min in a refrigerator at 5.0°C. The left foreleg of each larvae was then amputated at the base of the coxa using a surgical scalpel. The larvae were then isolated in plastic Petri dishes (4.5 cm diam) with their maternal lineage marked, and provisioned daily with *ad libitum* microencapsulated beads of artificial diet (Tan *et al*., 2014). Control larvae were treated in exactly the same way except that no limb was amputated. We recorded developmental time, the number of larvae that pupated, the fresh mass of pupae on their second day of pupation, and the number and sex of successfully emerging adults. Treatment adults were divided into two groups according to whether or not they had regenerated the amputated limb during pupation. Emerged adults were isolated in Petri dishes (as above) and fed *ad libitum* artificial diet, refreshed daily. Each adult was weighed on the day of emergence, prior to feeding, and again 7 days post-emergence. Since more melanic individuals (black elytra with variable numbers of red spots) were represented in the regenerated group than were succinic individuals (orange/red elytra with variable numbers of black spots), and melanism is a criterion for mate choice that varies seasonally in this species (Wang *et al*., 2009, 2012), it was decide to use only melanic forms in subsequent mating tests.

Mating observations – no choice

Three mating treatments were established ($n = 50$ replications per treatment) when adults were 10 days old: (1) regenerated ♂ × control ♀, (2) unregenerated ♂ × control ♀ and, (3) control ♂ × control ♀. Beetles were paired in Petri dishes with *ad libitum* diet beads, ensuring that all pairings were nonsiblings, and directly observed to record time to copula and duration of copula. Upon termination of copula, the male was removed and female held in isolation with *ad libitum* diet beads refreshed daily for the duration of her life. We recorded the total eggs laid by each female, the proportion hatching, and her longevity (days from emergence to death).

Mating observations – choice

Another cohort of 300 larvae were reared from the offspring of five females and prepared for the experiment as described above. Once they molted to the fourth instar, 150 were operated on in the same manner as described above. Mating arenas were prepared in Petri dishes (as above) with *ad libitum* diet beads ($n = 100$) in each. Each replicate consisted of a Petri dish containing one male of each type (regenerated, unregenerated, and control), selected to be approximately similar in size to prevent any possibility of size-based effects on male mating success. Small (1.0 mm^2) color-coded squares of adhesive plastic film were affixed to the left elytra of each male so that control and regenerated males could be distinguished. A single, control female, non-sibling to all males, was then introduced to the dish and directly observed until she copulated. We recorded the type of male selected by the female, the time to copula, and the duration of copula. Following termination of copula (disengagement of genitalia), the males were removed and all reproductive data were recorded for the female (as in the previous experiment) for the duration of her life.

Performance of the F1 generation

A series of ca. 500 larvae were reared from 5 to 6 egg clusters collected from each of the three no-choice mating treatments. The first instar larvae were isolated in plastic Petri dishes (as above) and supplied with *ad libitum* diet beads until they pupated. Following emergence of adults, the duration of development and survival (%) of each developmental stage was recorded. An additional 200 larvae were reared from each no-choice paternity treatment (in five cohorts of 40 individuals) and, within 24 h of molting to the fourth instar, a series of 150 larvae (selected to be of approximately similar size) each had a left foreleg amputated (as above). All larvae were then reared on *ad libitum* diet beads, refreshed daily, until they emerged as adults, which were then assessed for limb regeneration.

Data analysis

All data passed tests for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) and were analyzed by ANOVA. A 2-way ANOVA was used to test for interactions between maternal lineage and treatment. When three or more means were compared, they were separated by the Bonferroni test ($\alpha = 0.05$). Mating frequencies in 3-way choice tests and frequencies of melanism in the F1 generation were analyzed using the Chi-square (χ^2), Goodness-of-fit test.

Results

Limb amputation

Of the 150 larvae subjected to foreleg amputation, 124 (82.7%) survived to emerge as viable adults and, of these, 93 (75.0%) regenerated the severed leg (42♀♀, 51♂♂), whereas 31 (25%) did not (15♀♀, 16♂♂). Of regenerated individuals, 64.5% were melanic, compared to 45% of non-regenerates; control phenotypes were 67% melanic. Pupae that survived the treatment were heavier 2 days post-pupation than were control pupae, and both were heavier than pupae that died ($F_{2,190} = 39.38$, $P < 0.001$, Bonferroni $\alpha = 0.05$). Regenerating beetles spent, on average, 4–5 h longer in the pupal stage than did controls, whereas unregenerating beetles pupated almost half a day earlier (table 1). Regenerated males were heavier than unregenerated males on both weighing dates ($F_{2,85} = 5.1$, $P = 0.008$ and $F_{2,85} = 13.9$, $P < 0.001$, respectively, fig. 1a). The same pattern held true for females ($F_{2,76} = 6.6$, $P = 0.002$ and $F_{2,85} = 21.4$, $P < 0.001$, respectively) except that on the second sampling date, regenerated females were heavier than control females, that were in turn heavier than unregenerated females (fig. 1b). Regenerating beetles also gained more weight during their first week of adult life (mean ± SE = 5.7 ± 0.25 mg) than did non-regenerating beetles (mean ± SE = 4.5 ± 0.41 mg; $F_{2.164} = 3.60$, $P = 0.030$), with controls intermediate (mean ± SE = 5.1 ± 0.32 mg).

Table 1. Mean (±SE) pupation times and fresh weights 2 days post-pupation for *H. axyridis* adults that either had a single foreleg amputated in the fourth larval instar and regenerated the leg following pupation (regenerated) or did not (unregenerated), or were unoperated (control). Values bearing the same letter were not significantly different within columns (Bonferroni, $\alpha = 0.05$).

Beetle type	Pupation time (day)	Pupal wt. (mg)
Regenerated	4.2 ± 0.02 a	28.8 ± 0.42 a
Unregenerated	3.8 ± 0.02 c	26.8 ± 0.40 b
Control	4.0 ± 0.02 b	26.2 ± 0.22 b
F	88.7	10.5
df	2,164	2,164
P	<0.001	<0.001

Mating observations – no choice

There was no significant interaction between 'maternal lineage' and 'treatment' for time to copula ($F_{8,135} = 0.38$, $P = 0.927$), fecundity ($F_{8,135} = 0.16$, $P = 0.996$), or egg fertility ($F_{8,135} = 0.16$, $P = 0.995$), but the interaction term was significant for duration of copula ($F_{8,135} = 3.28$, $P = 0.002$). Furthermore, there was no significant main effect of maternal lineage on time to copula ($F_{4,135} = 0.77$, $P = 0.544$), fecundity ($F_{4,135} = 1.21$, $P = 0.308$), or egg fertility ($F_{4,135} = 0.355$, $P = 0.840$), but there was on duration of copula ($F_{4,135} = 4.69$, $P = 0.001$). The time to copulation was shortest for females provided with regenerated mates and longest for those provided with unregenerated males, with control males intermediate (table 2). Duration of copula was similar for control and regenerated males, but was reduced by about 40% for unregenerated males. Females paired with regenerated males obtained the highest fecundity, 20% higher than females paired with control males, whereas fecundity was 30% lower for females paired with unregenerated males. There was no significant effect of treatment on egg fertility.

Mating observations – choice

Females preferred to mate with regenerated males, selecting them 53 of 100 trials, compared to 20 unregenerated males and 25 control males ($\chi^2 = 19.18$, 2 df, $P < 0.001$). There

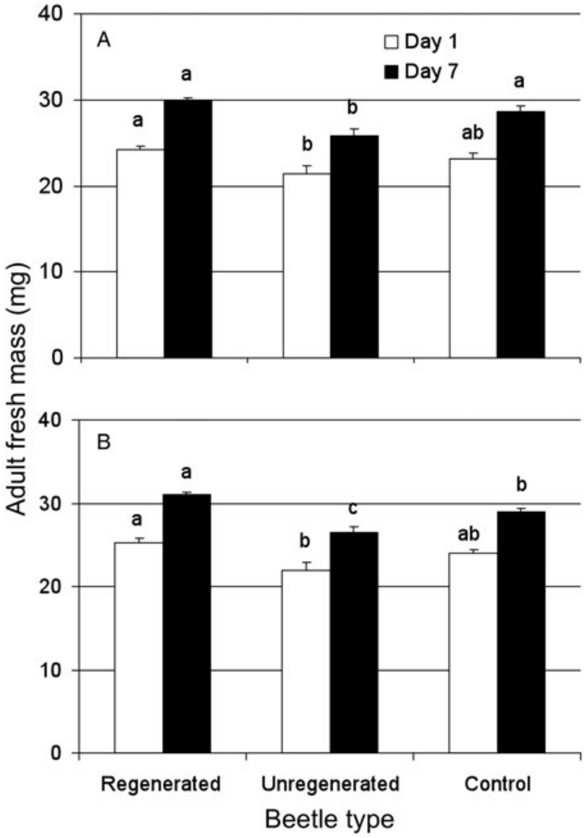

Fig. 1. Mean (+SE) fresh body weights of *H. axyridis* males (A) and females (B) on their first (open columns) and seventh (closed columns) days of adult life following regeneration of a single foreleg amputated in the fourth instar (regenerated) or not (unregenerated) or unoperated (control). Columns bearing different letters were significantly different from others of the same type (Bonferroni, α = 0.05).

were no significant interactions between maternal lineage and treatment for duration of copula ($F_{6,98} = 1.98$, $P = 0.078$), female fecundity ($F_{6,98} = 0.31$, $P = 0.928$), or egg fertility ($F_{6,98} = 0.84$, $P = 0.540$). Also, there were also no significant main effects of maternal lineage on duration of copula ($F_{4,98} = 0.67$, $P = 0.615$), female fecundity ($F_{4,98} = 2.15$, $P = 0.081$), or egg fertility ($F_{4,98} = 2.11$, $P = 0.087$). Duration of copula was about 4% lower in pairings with regenerated males compared to control males, but 46% lower in pairings with unregenerated males (table 3). Fecundity was similar for females that mated with control and regenerated males, but was reduced by about 25% for females that mated with unregenerated males. However, egg fertility was highest for females choosing regenerated males, with no difference between females choosing control and unregenerated males.

Performance of the F1 generation

The 2-way ANOVA revealed no significant interactions between maternal lineage and treatment for male body mass ($F_{6,88} = 2.27$, $P = 0.086$) or female body mass ($F_{6,112} = 1.30$, $P = 0.277$) and there were no significant main effects of maternal lineage for male ($F_{4,88} = 2.27$, $P = 0.086$) or female body mass ($F_{4,112} = 2.27$, $P = 0.086$). Progeny sired by control males had significantly faster development than progeny of operated males, regenerated or not, in the critical egg, fourth instar and pupal stages, leading to faster larval and total developmental periods (table 4). There were no significant interactions between maternal lineage and treatment for eclosion time ($F_{8,197} = 0.40$, $P = 0.920$), duration of the fourth instar ($F_{8,170} = 1.50$, $P = 0.161$), pupation time ($F_{8,161} = 0.38$, $P = 0.932$) or total developmental time ($F_{8,161} = 1.07$, $P = 0.388$) and no main effects of lineage on eclosion time ($F_{4,197} = 0.63$, $P = 0.641$), pupation time ($F_{4,161} = 1.01$, $P = 0.405$), or total developmental time ($F_{4,161} = 0.28$, $P = 0.888$), although there was a main effect of maternal lineage on duration of the fourth instar ($F_{4,170} = 4.28$, $P = 0.003$). Progeny sired by unregenerated males were less likely to survive from egg to adult than progeny of either regenerated or control males (table 4). Adult fresh mass was highest for progeny of control males, followed by progeny of regenerated males, and lowest for progeny of unregenerated males (fig. 2), regardless of whether sons ($F_{2,117} = 23.21$, $P < 0.001$) or daughters ($F_{2,93} = 15.17$, $P < 0.001$) were considered.

When subjected to leg amputation early in the fourth instar, progeny sired by regenerated males were significantly more likely to survive leg amputation than were those sired by control progeny, which in turn were more likely to survive amputation than those sired by unregenerated males ($F_{2,12} = 17.5$, $P < 0.001$, fig. 3). Furthermore, rates of limb recovery were similar for progeny sired by regenerated and control males, but were about two-thirds lower for progeny sired by unregenerated males ($F_{2,12} = 134.7$, $P < 0.001$). Progeny of regenerated males were 78.2% melanic, which was twice the frequency of melanism in progeny of unregenerated males (39.5%) or control males (38.3%; $\chi^2 = 20.84$, 2 df, $P < 0.001$).

Discussion

Amputation of a single foreleg had negative impacts on beetle development, supporting our initial hypothesis, and regenerating beetles were less impacted than non-regenerating beetles, supporting our second hypothesis. Beetles that successfully regenerated forelegs pupated at heavier weights than did control or non-regenerating beetles, suggesting that they consumed more food prior to pupation (not quantified), presumably due to the resource demands of regeneration. The longer time spent by regenerating beetles in the pupal stage relative to controls can be construed as a cost of regeneration. However, regeneration was also associated with greater weight gain during maturation, i.e., the first week of adult life, compared to non-regeneration, suggesting that regenerative capacity was associated with high voracity in both larval and adult stages. The larval ecology of *H. axyridis* involves strong intraguild interactions with many other predatory arthropods on the aphid colonies where they develop (Michaud, 2002; Pell *et al.*, 2008; Katsanis *et al.*, 2013). Clearly, the ability to regenerate a lost limb will yield a selective advantage for predators that must develop in an environment full of dangerous competitors. However, it is also possible that limb regeneration is not under direct selection itself, but is linked to other traits that are (e.g., high egg fertility). Further investigation is thus warranted to determine what other biological traits might be linked to regenerative ability in both males and females.

Our third hypothesis was also supported; females clearly favored mating with regenerating males over controls or non-

Table 2. Mean (±SE) time to copula, duration of copula, and reproductive success of female *H. axyridis* mated to males in a no-choice situation that either had a single foreleg amputated in the fourth larval instar and regenerated the leg following pupation (regenerated) or did not (unregenerated), or an unoperated, control male (control). Values bearing the same letter were not significantly different within columns (Bonferroni, $\alpha = 0.05$).

Sire	Time to copula (min)	Duration of copula (min)	Fecundity (no. eggs)	Fertility (% hatching)
Regenerated	95.0 ± 1.7 c	687.0 ± 3.1 a	260.6 ± 3.4 a	68.2 ± 1.1 a
Unregenerated	266.7 ± 2.3 a	400.8 ± 5.0 b	149.4 ± 2.8 c	65.3 ± 1.6 a
Control	231.1 ± 4.9 b	698.2 ± 10.2 a	215.0 ± 1.8 b	71.9 ± 1.0 a
F	776.8	615.0	417.1	2.65
df	2,147	2,147	2,147	2,147
P	<0.001	<0.001	<0.001	0.074

Table 3. Mean (±SE) duration of copula, and reproductive success of female *H. axyridis* given a choice of males that either had a single foreleg amputated in the fourth instar and regenerated the leg during pupation (regenerated) or did not (unregenerated), or an unoperated, control male (control). Values bearing the same letter were not significantly different within columns (Bonferroni, $\alpha = 0.05$).

Sire	*n*	Duration of copula (min)	Fecundity (no. eggs)	Fertility (% hatching)
Regenerated	53	664.9 ± 2.1 b	223.1 ± 2.1 a	81.7 ± 1.0 a
Unregenerated	20	372.7 ± 2.6 c	169.2 ± 2.4 b	63.3 ± 2.2 b
Control	25	691.4 ± 2.4 a	226.6 ± 2.8 a	67.5 ± 4.3 b
F		3775.7	119.7	20.0
df		2,95	2,95	2,95
P		<0.001	<0.001	<0.001

Table 4. Mean (±SE) developmental parameters for progeny of *H. axyridis* females mated to males that either regenerated a foreleg amputated in the fourth instar (regenerated) or did not (unregenerated), or an unoperated, control male (control).

Parameter	Sire			*F*	df	*P*
	Regenerated	Unregenerated	Control			
Eclosion (day)	3.8 ± 0.03 a	3.9 ± 0.04 a	3.4 ± 0.06 b	31.8	2,209	<0.001
Larval development (day)	16.2 ± 0.09 a	16.4 ± 0.10 a	15.6 ± 0.08 b	20.0	2,182	<0.001
Pupation time (day)	4.1 ± 0.02 a	4.2 ± 0.02 a	4.0 ± 0.03 b	17.7	2,173	<0.001
Total development (day)	20.4 ± 0.09 a	20.6 ± 0.10 a	19.6 ± 0.09 b	27.8	2,173	<0.001
Immature survival (%)	87.6 a	70.4 b	87.0 a	[1]		<0.05

[1] Pairwise, two-tailed test of proportions, weighted by sample size.

Means followed by different letters were significantly different within rows (Bonferroni, $\alpha = 0.05$).

regenerators. Females assigned unregenerated males in the no-choice experiment took longer to accept them and obtained shorter copulations and lower fecundities relative to either control or regenerated males. Thus, failure to regenerate a leg was associated with a reduction in male fitness that was not suffered by males able to regenerate. Females were also able to distinguish regenerated males from control males, despite their superficial similarity, coupling with them more than twice as quickly in the no-choice experiment, and more often than expected by chance in the choice experiment. Females that selected unregenerated males received shorter copulations and obtained lower fecundities than those selecting control or regenerated males. Females selecting regenerated males obtained higher egg fertility than females selecting control males, although their mean fecundity was similar and their duration of copula slightly shorter. Thus, regenerative capacity appeared to be associated with a suite of male traits that were both preferred by females, and that improved their reproductive performance, raising the possibility that it is a byproduct of selection acting on other traits associated with male fitness or reproductive success.

The mechanism by which females recognized regenerated males was not resolved in these experiments. Since regenerated beetles tended to have higher pupal weights, it might be argued that females responded to the larger size of regenerated males. Ueno *et al.* (1998) found that *H. axyridis* males collected in copula in central Japan tended to be larger than those collected as solitary beetles, although this was true only for non-melanic forms and could have resulted from large males being more successful in overpowering reluctant females. For example, Perry *et al.* (2009) found that starved female *Adalia bipunctata* L. were just as likely to resist matings from large males as from small ones, although the former were more effective at countering this resistance. To our knowledge, size has not yet been unequivocally established as a criterion for female choice in any coccinellid species. We suggest that qualitative differences in biochemistry associated with regenerative capacity are more likely to explain our results than differences in male size. Various species-specific alkaloids are endogenously produced by coccinellids (Daloze *et al.*, 1995; Laurent *et al.*, 2002; Haulotte *et al.*, 2012). Although used primarily in defense, these compounds serve multiple ecological

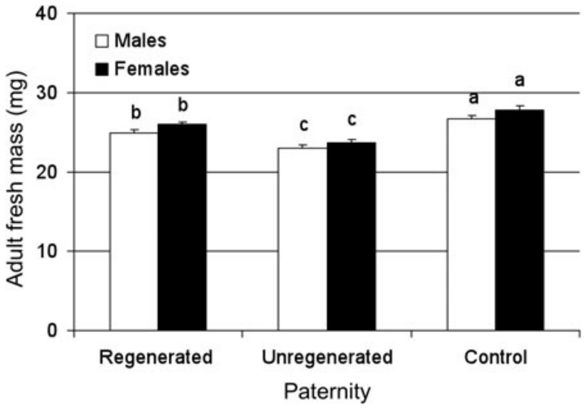

Fig. 2. Mean (+SE) fresh body weights of *H. axyridis* male (open columns) and female (closed columns) progeny sired by males of three different types: those that successfully regenerated a single foreleg amputated in the fourth instar (regenerated) or did not (unregenerated) or unoperated (control). Columns bearing different letters were significantly different from others of the same sex (Bonferroni, α = 0.05).

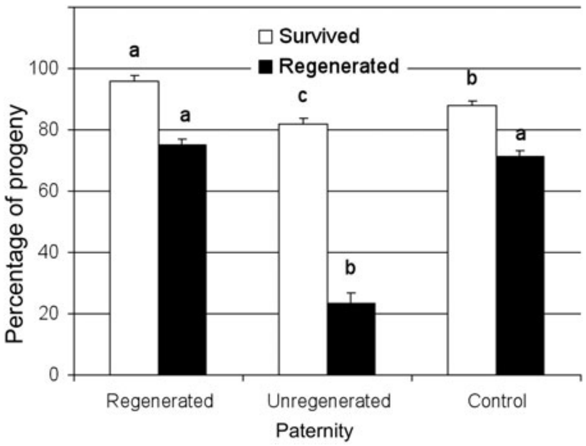

Fig. 3. Mean (+SE) percentages of *H. axyridis* progeny that survived (open columns) and regenerated a single foreleg amputated in the fourth instar (closed columns) when sired by males of three different types: those that successfully regenerated a single foreleg amputated in the fourth instar (regenerated) or did not (unregenerated) or unoperated (control). Columns bearing different letters were significantly different from others of the same type (Bonferroni, α = 0.05).

signaling functions including oviposition deterrence (Hemptinne & Dixon, 2000; Martini *et al.*, 2009) and sexual identity (Hemptinne *et al.*, 1998). Evidence of kin-recognition in *H. axyridis* and other coccinellids (Joseph *et al.*, 1999; Michaud, 2003; Martini *et al.*, 2013) suggests that these insects are sensitive to subtle, qualitative differences in semiochemistry that could also facilitate individual recognition and assessment of physiological condition. Regenerative capacity was also positively associated with melanism in both generations, suggesting a pleiotropic influence of melanism on processes involved in regeneration. Melanin has pleiotropic effects on multiple biological functions in both vertebrates and invertebrates, including developmental processes (True, 2003). For example, previous work on *H. axyridis* has shown that alkaloid profiles can vary as a function of degree of melanization (Bezzerides *et al.*, 2007) and that melanism itself is a flexible criterion for mate choice in this species (Osawa & Nishida, 1992; Wang *et al.*, 2009, 2013).

The trauma of amputation inflicted costs on males that extended to the fitness of their progeny, supporting our fourth hypothesis. Progeny of amputated males had slower development and lower adult mass compared to control males, regardless of whether they regenerated the limb or not, indicating that amputation negatively impacted (or diminished) normal paternal effects. Paternal effects in the Coccinellidae can vary with male age and condition but are normally beneficial, typically enhancing egg fertility, accelerating progeny development, and increasing adult body size (e.g., Michaud *et al.*, 2013; Mirhosseini *et al.*, 2014). Deleterious genetics appeared to be associated with a lack of regenerative ability, as progeny of unregenerated paternity had lower survival than progeny of control paternity, and lower adult body mass than progeny of regenerated paternity, supporting our fifth hypothesis.

Female reproductive data support a linkage between regeneration and male fitness; females obtained more viable offspring by mating with regenerated males, even though their developmental phenotypes were slightly impaired. Since regeneration is not fixed within the population, tradeoffs likely exist between regenerative capacity and other traits linked to fitness. A better understanding of these tradeoffs will require data on the relative fitness of regenerators versus non-regenerators with and without the trauma of leg loss, under both laboratory and natural conditions. Experimentally, this would require some molecular marker of regenerative capacity that, if recoverable from the egg chorion, could be used to distinguish regenerators in the larval stage without subjecting them to amputation. However, regenerative capacity had relatively high heritability; when offspring of regenerated males were themselves subjected to foreleg amputation, they had higher rates of survival than offspring of either control or unregenerated males. Although progeny of regenerated and control beetles exhibited similar regenerative abilities, progeny of unregenerated beetles had only one-third the probability of replacing the limb.

One caveat of our experiments is that the left foreleg was always amputated, and it is possible the regenerative process may have a lateral bias. Behavioral lateralization is well recognized in higher vertebrates (Rogers & Andrew, 2002) and evidence is emerging of its importance in insect behavior as well (Frasnelli *et al.*, 2012). For example, Benelli *et al.* (2015) used high-speed video recording to demonstrate population-level behavioral asymmetries in Mediterranean fruit flies, *Ceratatis capitata* (Wiedemann), characterized by the preferential utilization of left-side body parts (forelegs and wings) in aggressive same-sex interactions. If there exist behavioral asymmetries in the mating behavior of *H. axyridis*, amputation of the right foreleg may not yield the same results as amputation of the left, assuming regenerated forelegs differ in functionality. Thus, limb-regenerating *H. axyridis* beetles could represent a useful model system for exploring the lateralization of biological and behavioral processes in tandem.

Conclusion

A large majority of *H. axyridis* larvae subjected to limb amputation early in the fourth instar were able to regenerate the limb during pupation, although they required additional resources to do so (increased consumption and more time in the pupal stage). Regenerated males were preferred as mates by females and endowed them with higher reproductive success, although paternal effects on progeny were compromised. To date, research on limb regeneration in holometabolous insects has been limited to examination of mechanisms in larvae of flour beetles and silkworms, species unable to regenerate legs beyond larval stages. Our findings suggest that pupal limb regeneration in the Coleoptera, and perhaps other holometabolous insect orders, might be more widespread than currently recognized. The fact that the *H. axyridis* population remains polymorphic for regenerative capacity and suggests that it may entail fitness tradeoffs, an important topic for future investigation. This is the first report of complete limb regeneration in the pupal stage by a holometabolous insect, and the first known example of sexual selection for regenerative capacity.

Acknowledgements

This study was funded through the Major State Basic Research Development Program of China (973 Program (No. 2013CB127605), Beijing Nova program (No. Z121105002512039) and Special Fund for Agro-scientific Research in the Public Interest (No. 201303108), and the Beijing NOVA program (No. Z121105002512039). This is contribution No. 15-157-J of the Kansas Agricultural Experiment Station.

References

Alibardi, L. (2012) Observations on FGF immunoreactivity in the regenerating tail blastema, and in the limb and tail scars of lizard suggest that FGFs are required for regeneration. *Journal of Zoology* **142**, 23–38.

Benelli, G., Donati, E., Romano, D., Stefanini, C., Messing, R.H. & Canale, A. (2015) Lateralisation of aggressive displays in a tephritid fly. *Science of Nature* (in press) DOI: 01/2015; DOI: 10.1007/s00114-014-1251-6.

Bezzerides, A.L., McGraw, K.J., Parker, R.S. & Husseini, J. (2007) Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. *Behavioral Ecology and Sociobiology* **61**, 1401–1408.

Brockes, J.P. & Gates, P.B. (2014) Mechanisms underlying vertebrate limb regeneration: lessons from the salamander. *Biochemical Society Transactions* **42**, 625–630.

Cuervo, R., Hernandez-Martinez, R., Chimal-Monroy, J., Merchant-Larios, H. & Covarrubias, L. (2012) Full regeneration of the tribasal *Polypterus* fin. *Proceedings of the National Academy of Sciences* **109**, 3838–3843.

Daloze, D., Braekman, J.C. & Pasteels, J.M. (1995) Ladybird defence alkaloids: structural, chemotaxonomic and biosynthetic aspects (Col.: Coccinellidae). *Chemoecology* **5**/**6**, 173–183.

Frasnelli, E., Vallortigara, G. & Rogers, L. (2012) Left-right asymmetries of behaviour and nervous system in invetebrates. *Neuroscience and Biobehavioral Reviews* **36**, 1273–1291.

Grafen, A. (1990*a*) Sexual selection unhandicapped by the Fisher process. *Journal of Theoretical Biology* **144**, 475–516.

Grafen, A. (1990*b*) Biological signals as handicaps. *Journal of Theoretical Biology* **144**, 517–546.

Haulotte, E., Laurent, P. & Braekman, J.C. (2012) Biosynthesis of defensive coccinellidae alkaloids: incorporation of fatty acids in adaline, coccinelline, and harmonine. *European Journal of Organic Chemistry* **10**, 1907–1912.

Hemptinne, J.L. & Dixon, A.F.G. (2000) Defence, oviposition and sex: semiochemical parsimony in two species of ladybird beetles (Coleoptera, Coccinellidae)? A short review. *European Journal of Entomology* **97**, 443–447.

Hemptinne, J.L., Lognay, G. & Dixon, A.F.G. (1998) Mate recognition in the two-spot ladybird beetle, *Adalia bipunctata*: role of chemical and behavioural cues. *Journal of Insect Physiology* **44**, 1163–1171.

Joseph, S.B., Snyder, W.E. & Moore, A.J. (1999). Cannibalizing *Harmonia axyridis* (Coleoptera: Coccinellidae) larvae use endogenous cues to avoid eating relatives. *Journal of Evolutionary Biology* **12**, 792–797.

Karuppanan, U. (1998) Regeneration in the limbs of mantids (Dictyoptera: Mantidae). *Journal of Ecobiology* **10**, 27–36.

Katsanis, A., Babendreier, D., Nentwig, W. & Kenis, M. (2013) Intraguild predation between the invasive ladybird *Harmonia axyridis* and non-target European coccinellid species. *BioControl* **58**, 73–83.

Knobloch, C.A. & Steel, C.G.H. (1988) Interactions between limb regeneration and ecdysteroid titers in last larval instar *Rhodnius prolixus* (Hemiptera). *Journal of Insect Physiology* **34**, 507–514.

Koch, R.L. (2003) The multicolored Asian lady beetle, *Harmonia axyridis*: a review of its biology, uses in biological control, and non-target impacts. *Journal of Insect Science* **3**, 32.

Kumar, A., Nevill, G., Brockes, J.P. & Forge, A. (2010) A comparative study of gland cells implicated in the nerve dependence of salamander limb regeneration. *Journal of Anatomy* **217**, 16–25.

Lakes, R. & Mucke, A. (1989) Regeneration of the foreleg tibia and tarsi of *Ephippiger ephippiger* (Orthoptera: Tettigoniidae). *Journal of Experimental Zoology* **250**, 176–187.

Laurent, P., Braekman, J.C., Daloze, D. & Pasteels, J.M. (2002) *In vitro* production of adaline and coccinelline, two defensive alkaloids from ladybird beetles (Coleoptera: Coccinellidae). *Insect Biochemical and Molecular Biology* **32**, 1017–1023.

Lee, A.K., Sze, C.C., Kim, E.R. & Suzuki, Y. (2013) Developmental coupling of larval and adult stages in a complex life cycle: insights from limb regeneration in the flour beetle, *Tribolium castaneum*. *Evodevo* **4**, 20. doi: 10.1186/2041-9139-4-20.

Li, H., Zhang, X.H. & Na, J. (2007) The hind leg regeneration in the nymphs of *Gryllus bimaculata*. *Chinese Bulletin of Entomology* **44**, 419–422.

Lobo, D., Beane, W.S. & Levin, M. (2012) Modeling planarian regeneration: a primer for reverse-engineering the worm. *PLoS Comparative Biology* **8**, e100248.

Martini, X., Haccou, P., Olivieri, I. & Hemptinne, J.L. (2009) Evolution of cannibalism and female's response to oviposition-deterring pheromone in aphidophagous predators. *Journal of Animal Ecology* **78**, 964–972.

Martini, X., Dixon, A.F.G. & Hemptinne, J.L. (2013) The effect of relatedness on the response of *Adalia bipunctata* L. to oviposition deterring cues. *Bulletin of Entomological Research* **103**, 14–19.

Michaud, J.P. (2002) Invasion of the Florida citrus ecosystem by *Harmonia axyridis* (Coleoptera: Coccinellidae) and asymmetric competition with a native ladybeetle, *Cycloneda sanguinea*. *Environmental Entomology* **31**, 827–835.

Michaud, J.P. (2003) A comparative study of larval cannibalism in three species of ladybird. *Ecological Entomology* **28**, 92–101.

Michaud, J.P., Bista, M., Mishra, G. & Omkar (2013) Sexual activity diminishes male virility in two *Coccinella* species:

consequences for female fertility and progeny development. *Bulletin of Entomological Research* **103**, 570–577.

Mirhosseini, M.A., Michaud, J.P., Jalali, M.A. & Ziaaddini, M. (2014) Paternal effects correlate with female reproductive stimulation in a polyandrous ladybird, *Cheilomenes sexmaculata*. *Bulletin of Entomological Research* **104**, 480–485.

Mitten, E.K., Jing, D. & Suzuki, Y. (2012) Matrix metalloproteinases (MMPs) are required for wound closure and healing during larval leg regeneration in the flour beetle, *Tribolium castaneum*. *Insect Biochemical and Molecular Biology* **42**, 854–864.

Murciano, C., Fernandez, T.D., Duran, I., Maseda, D., Ruiz-Sanchez, J., Becerra, J., Akimenko, M.A. & Mari-Beffa, M. (2002) Ray-interray interactions during fin regeneration of *Danio rerio*. *Developmental Biology* **252**, 214–224.

Nacu, E. & Tanaka, E. (2011) Limb regeneration: a new development? *Annual Review of Cell and Developmental Biology* **27**, 409–440.

Osawa, N. & Nishida, T. (1992) Seasonal variation in elytral colour polymorphism in *Harmonia axyridis* (the ladybird beetle): the role of non-random mating. *Heredity* **69**, 297–307.

Passamaneck, Y.J. & Martindale, M.Q. (2012) Cell proliferation is necessary for the regeneration of oral structures in the anthozoan cnidarian *Nematostella vectensis*. *BMC Developmental Biology* **12**, 34. doi: 10.1186/1471-213X-12-34.

Pell, J.K., Baverstock, J., Roy, H.E., Ware, R.L. & Majerus, M.E.N. (2008) Intraguild predation involving *Harmonia axyridis*: a review of current knowledge and future perspectives. *BioControl* **53**, 147–168.

Perry, J.C., Sharpe, D.M.T. & Rowe, L. (2009) Condition-dependent female remating resistance generates sexual selection on male size in a ladybird beetle. *Animal Behaviour* **77**, 743–748.

Rogers, J.L. & Andrew, R.J. (2002) Comparative vertebrate lateralization. Cambridge University. Press.

Roy, H.E. & Wajnberg, E. (2008) From biological control to invasion: the ladybird *Harmonia axyridis* as a model species. *BioControl* **53**, 1–4.

Satoh, A., Graham, G.M.C., Bryant, S.V. & Gardiner, D.M. (2008) Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (*Ambystoma mexicanum*). *Developmental Biology* **319**, 321–335.

Satoh, A., Bryant, S.V. & Gardiner, D.M. (2012) Nerve signaling regulates basal keratinocyte proliferation in the blastema apical epithelial cap in the axolotl (*Ambystoma mexicanum*). *Developmental Biology* **366**, 374–381.

Selin, N.I. (2003) Limb regeneration in male tanner crabs *Chionoecetes bairdi* and *Ch. opilio*. *Biologiya Morya (Vladivostok)* **29**, 198–201.

Shah, M., Namigai, E. & Suzuki, Y. (2011) The role of canonical Wnt signaling in leg regeneration and metamorphosis in the red flour beetle *Tribolium castaneum*. *Mechanisms of Development* **4**, 342–400.

Shock, B.C., Foran, C.M. & Stueckle, T.A. (2009) Effects of salinity stress on survival, metabolism, limb regeneration, and ecdysis in *Uca pugnax*. *Journal of Crustacean Biology* **29**, 293–301.

Singh, A., Kango-Singh, M., Parthasarathy, R. & Gopinathan, K. P. (2007) Larval legs of mulberry silkworm *Bombyx mori* are prototypes for the adult legs. *Genesis* **45**, 169–176.

Tan, X., Zhao, J., Wang, S. & Zhang, F. (2014) Optimization and evaluation of microencapsulated artificial diet for mass rearing the predatory ladybird *Propylea japonica* (Coleoptera: Coc-cinellidae). *Insect Science* doi: 10.1111/1744-7917.12098.

Tanaka, A., Ohtake-Hashiguchi, M. & Ogawa, E. (1987) Repeated regeneration of the German cockroach legs. *Growth* **51**, 282–300.

Truby, P.R. (1983) Blastema formation and cell division during cockroach limb regeneration. *Journal of Embryology and Experimental Morphology* **75**, 151–164.

True, J.R. (2003) Insect melanism: the molecules matter. *Trends in Ecology and Evolution* **18**, 640–647.

Ueno, H., Sato, Y. & Tusuchida, K. (1998) Colour-associated mating success in a polymorphic ladybird beetle, *Harmonia axyridis*. *Functional Ecology* **12**, 757–761.

Wang, S., Michaud, J.P., Zhang, R., Zhang, F. & Liu, S. (2009) Seasonal cycles of assortative mating and reproductive behaviour in polymorphic populations of *Harmonia axyridis* in China. *Ecological Entomology* **34**, 483–494.

Wang, S., Michaud, J.P., Tan, X.L., Murray, L. & Zhang, F. (2013) Melanism in a Chinese population of *Harmonia axyridis* (Coleoptera: Coccinellidae): a criterion for male investment with pleiotropic effects on behavior and fertility. *Journal of Insect Behavior* **26**, 679–689.

Wigglesworth, V.B. (1965) *The Principles of Insect Physiology*. 6th edn. 741 pp. London, Methuen.

Wolpert, L. (2011) Positional information and patterning revisited. *J Theor Biol.* **269**(1), 359–65.

Yanez-Rivera, B. & Mendez, N. (2014) Regeneration in the stinging fireworm *Eurythoe* (Annelida): lipid and triglyceride evaluation. *Journal of Experimental Marine Biology and Ecology* **459**, 137–143.

Zahavi, A. (1975) Mate selection – a selection for a handicap. *Journal of Theoretical Biology* **53**, 205–214.

Zahavi, A. & Zahavi, A. (1997) *The Handicap Principle: A Missing Piece of Darwin's Puzzle*. Oxford, Oxford University Press.