Leg Regeneration Trade-Offs in the Twostriped Walkingstick (Phasmatodea: Pseudophasmatidae)

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ABSTRACT

Appendage regeneration is a unique tool to study resource allocation trade-offs; the resources allocated to grow an appendage a second time can come at the expense of other structures. We studied the effects of both front and hind leg regeneration on a suite of traits in Anisomorpha buprestoides (Stoll) (Phasmatodea: Pseudophasmatidae), the twostriped walkingstick. This species is ideal for resource allocation questions because it has a relatively long life history as well as a large group of traits to measure for any potential trade-offs (including large defensive glands in the thorax that are used to deter predators). For females, there were no apparent trade-offs with respect to antennae, eyes, defensive glands, nonregenerated legs, cerci, genital plates, and/or body size. In males, there were no also no apparent trade-offs with respect to antennae, eyes, defensive glands, nonregenerated legs, cerci, or testes, but males that regenerated a leg were larger (but not heavier) than nonregenerating males. Future work at the physiological level will hopefully elucidate the nature of acquisition-allocation trade-offs in phasmids, and how such trade-offs may have shaped their evolution.

KEY WORDS phasmids, stick insects, regeneration, trade-offs, life history

Trade-offs are a very inherent and thoroughly studied foundation of evolutionary biology (Darwin 1859, Roff 1992, Stearns 1992, Fairbairn 1994, Futuyma 1998, Trumbo 1999, Reznick et al. 2000, Roff and Fairbairn 2007). One important type of trade-off is the resource allocation trade-off, in which several traits compete for the same pool of resources during development. Our best empirical examples of these types of resource allocation trade-offs have been measured by three nonmutually exclusive approaches: 1) artificial selection experiments over several generations that select for one trait and measure the response in another trait (e.g., stalk-eyed flies [Wilkinson 1993, Fry 2006]), 2) measure a suite of traits from individuals of a single species (or group of closely related species) that show a phenotypicism with respect to an exaggerated aspect of their morphology (e.g., major and minor males [Kawano 1997, Emlen 2001, Tomkins et al. 2005], long- and short-winged crickets [Roff 1986, Zera and Brink 2000, Zera and Harshman 2001]), or 3) by experimentally manipulating the phenotype of an individual in the laboratory and then measuring its effects on other traits (e.g., butterfly wings [Klingenberg and Nijhout 1998, Nijhout and Emlen 1998, Emlen and Nijhout 2000]). With respect to the second and third approaches, appendage regeneration is a novel twist to measuring trait-trait trade-offs because many of these animals naturally invest in a trait not once, but twice.

Previous research in reptiles, amphibians, fish, crustaceans, echinoderms, insects, and arachnids has demonstrated how costly the regeneration process can be (Bliss 1960; Maiorana 1977; Bulliere and Bulliere 1985; Lawrence and Larraín 1994; Juanes and Smith 1995; for review, see Maginnis 2006a). In phasmids specifically, leg regeneration has shown several different types of trade-offs depending on morphology. The regrowth of a lost leg in the winged Sipyloidea sipylius (Westwood) stunted wing growth, and in the wingless Caraunius morosus (Westwood) leg regeneration decreased fecundity and reduced overall body size (Maginnis 2006b, T.L.M. and D. Emle, in review). Thus, this one developmental process can show different trait-trait trade-offs in different phasmid morphologies.

However, it is currently unknown how pervasive leg regeneration trade-offs are in phasmids and exactly how they may vary from species to species. Anisomorpha buprestoides (Stoll) (Phasmatodea: Pseudophasmatidae), the twostriped walkingstick, is quite different from previously studied phasmids. First, it has an 8-mo developmental period; complete development takes more than twice as long as S. sipylius and C. morosus. Moreover, A. buprestoides is approximately half the size of both S. sipylius and C. morosus (female A. buprestoides average 3.5 cm in length, and female S. sipylius and C. morosus average 7–9 cm in length). Under these circumstances, it is possible individuals could acquire enough time or food to alleviate any resource allocation trade-offs that were predicted to arise due to leg regeneration. Second, A. buprestoides
exhibits sexual reproduction and both sexes readily occur; in many phasmid species (including S. sipylus and C. morosus), the males are either extremely rare or species members exist only as females and rely on parthenogenesis for reproduction (Brock 1999). A. buprestoides also exhibits strong sexual dimorphism; the males are much more slender and approximately half the length of females (males average 3.5 cm and 0.2 g, and females average 6.0 cm and 1.6 g; see Results). These variations between the sexes raise the intriguing possibility that males and females manifest trade-offs in different ways. And third, although it is wingless like the previously studied C. morosus, it has a unique defensive mechanism unknown in any other genera of phasmids; as a fairly terrestrial (rather than arboreal) phasmid, A. buprestoides discharges a burning, pungent mist from one or both of its thoracic glands facing predation (MacAtee 1918, Albert 1947, Eisner 1965, Eisner and Meinwald 1966, Carlberg, 1985). The defensive chemical is a type of cyclopentanoid monoterpene called anisomorphal, from which the genus name is derived (Meinwald et al. 1962, Eisner and Meinwald 1966).

Given these dramatic differences, A. buprestoides provides an excellent opportunity to further explore how pervasive leg regeneration trade-offs are in phasmids, and how they may vary between the sexes, and among different life histories, and different gross morphologies. In addition, previous studies have only explored trade-offs associated with hind leg regeneration. Field studies have documented how all six legs occur; in many phasmid species (including A. buprestoides) and C. morosus, parthenogenesis for reproduction (Brock 1999), the males are either extremely rare or species members exist only as females and rely on parthenogenesis for reproduction (Brock 1999).

Materials and Methods

Twelve adult A. buprestoides (six mating pairs) were collected July 2007 in Austin, TX. The eggs from those adults hatched in early October 2007, and 118 second-instar nymphs were randomly placed into one of three treatment groups, because the sexes were indistinguishable at the time. Regenerated legs require three molts; the first molt after leg loss produces a leg approximately one fourth the normal size; the second molt after leg loss produces a leg approximately one half the normal size; the third molt after leg loss produces a leg nearly normal in size (T.L.M., unpublished data). Individuals of each treatment group were kept together in one large cage (three cages total, each with four large pieces of debris as habitat), misted with water once a day, and fed ad libitum (Ligustrum spp.) until they matured in late May.

Live adults were weighed on an analytical balance and subsequently anesthetized with CO₂ for 2 min. Females were immediately dissected, and photographs of their defensive glands were taken. Males were also immediately dissected, first for testes weight and then for gland photography; testes were removed with forceps and weighed together on a Mettler Toledo microbalance. Unfortunately, there was no measure of female fecundity in this study; we began measuring traits as soon as possible after the majority of individuals matured (to be consistent across the sexes) and intended to weigh ovaries or ovarian tissues, but some of the females had already begun to produce mature eggs via parthenogenesis. Thus, a consistent measure of female fecundity across all individuals was not possible. Gland photographs, as well as all the photographs described below, were taken with a stereomicroscope (Leica, Deerfield, IL) equipped with an ocular micrometer.

After all the dissected individuals were dried in a laboratory drying oven, the antennae, all legs and cerci were removed from the body (leaving the head, thorax, and abdomen intact). Legs were removed at the coxa-femur junction and antennae were removed at the junction between the most basal segment and the head. Individual cerci are composed of one sclerotized piece and were simply pulled from the end of the abdomen with forceps (see Littig 1942 for an anatomical description). Eyes and female genital plates were not removed from the body; secondary close-up photographs were used for measurements. All photographed traits (body size, antennae, eyes, glands, legs, cerci, and genital plates) were measured with ImageJ (1.41o; National Institutes of Health, Bethesda, MD). Body size, antennae, and all legs were measured for length (at the longest portion); eyes, glands, cerci and female genital plates were measured for area (around the largest perimeter).

Several statistical analyses were done to test for resource allocation trade-offs. Two t-tests (assuming unequal variance) between the control and regenerated treatment groups tested for trade-offs associated with body size. To test for the effects of regeneration on the regenerated leg, two analyses were performed: paired t-tests between the right and left legs within the regenerated treatment groups, and t-tests (assuming unequal variance) between the right legs of the control group and the right legs of the treatment groups. To test for trade-offs with other paired structures (e.g., antennae, eyes, glands, and cerci), the data were first
tested for differences between the right and left trait with a paired t-test (assuming unequal variance). This is because noncontrol individuals regenerated a right leg, we explored whether the right antennae, eyes, glands or cerci were significantly different than those on the left. For all four paired traits in each of the three treatment groups, the right and left sides did not significantly differ (all values $P > 0.37$), so the measurements of the right and left trait were averaged and a single measurement of antennae, eyes, glands and cerci were used for each individual.

Two analyses of covariance (ANCOVA) were used to test for effects of leg regeneration on antennae length, eye area, gland area, cerci area, testes weight, and genital plate area: one analysis with body size as the covariate and one analysis with body mass as the covariate. For all measurements, the [treatment $\times$ body size/mass] interaction terms were not significant. To better test for treatment effects on the $y$-intercept, ANCOVAs were performed using body size and body mass measurements that were standardized to a mean of 0 (e.g., by subtracting the mean body size/mass from all values). This does not affect the slopes or interactions, but better tests for a shift in $y$-intercept in the middle of data range (instead of several standard deviations outside of this range, if actual body size or body mass measurements were included and the $y$-intercepts were located at a size value of 0).

Results

Breeding and Behavior Results. One hundred and one of the 118 nymphs survived to maturity (85.6% survivorship). Of those 101 individuals, 14 (13.9%) lost an additional leg at an unknown point in the experiment (presumably due to molting complications) and were excluded from the analysis, and three individuals were still in their penultimate instar by the time we began the dissections. Thus, the final sample size was 84 individuals: 39 males ($N_0 = 16$, $N_{\text{front}} = 13$, $N_{\text{hind}} = 10$) and 45 females ($N_0 = 17$, $N_{\text{front}} = 12$, $N_{\text{hind}} = 16$). Because all individuals from each treatment group were kept in one large cage, the exact or average duration of each instar was not recorded. However, periodic measurements revealed A. buprestoides has approximately six nymphal instars; we were unable to determine whether females have one more molt than males, which occurs in some phasmids species (Brock 1999). The average ± SD length of all the adult females was $6.07 \pm 0.27$ cm, and average weight was $1.60 \pm 0.49$ g. The average length of all the adult males was $3.54 \pm 0.18$ cm, and average weight was $0.25 \pm 0.19$ g.

Regular observations during rearing revealed three interesting behaviors of A. buprestoides. First, individuals were highly gregarious during the day; almost all of the individuals were found piled on top of each other, hiding under the large pieces of debris. Second, individuals would rapidly tap their legs (usually their front legs) against the substrate upon disturbance, collectively sounding like a light and fast drum roll. And third, some males mate-guarded the females several instars before either sex was mature. Although mate guarding is a well-known behavior of adult phasmids (Bedford 1978, Sivinski 1980, Brock 1999), some males showed mate-guarding behavior three months before maturing into sexual adults.

Morphometric Results. As with all studied phasmids to date, regenerated legs were proportionately smaller than nonregenerated legs [t-test assuming unequal variance], and leg regeneration had no effect on other pairs of legs (single-factor ANCOVA, body size as the covariate). $N_0$, sample size for control treatment group; $N_{\text{front}}$, sample size for treatment group that regenerated a front right leg; and $N_{\text{hind}}$, sample size for treatment group that regenerated a hind right leg.

| Regenerated leg | Front legs | Mid legs | Hind legs |
|-----------------|------------|----------|-----------|
| $t$             | $F$        | $P$      | $F$       | $P$       |
| $N_0 = 16$      | $7.240$    | $0.001$  | $0.046$   |           |
| $N_{\text{front}} = 13$ | $P < 0.0001$ | $979$ | $0.532$ |           |
| $N_{\text{hind}} = 10$ | $0.0002$ | $0.791$ | $0.696$ | N/A |
| $N_0 = 16$      | $4.827$    | $0.072$  | $0.157$   |           |
| $N_{\text{front}} = 12$ | $0.0001$ | $0.106$ | $0.100$ | N/A |
| $N_{\text{hind}} = 10$ | $0.0001$ | $0.096$ | $0.055$ | N/A |
| $N_0 = 17$      | $4.854$    | $2.031$  | $2.906$   |           |
| $N_{\text{front}} = 13$ | $0.0001$ | $0.100$ | $0.100$ | N/A |
| $N_{\text{hind}} = 16$ | $0.0001$ | $0.096$ | $0.055$ | N/A |

Regenerated legs are proportionally smaller than nonregenerated legs (t-test assuming unequal variance), and leg regeneration had no effect on other pairs of legs (single-factor ANCOVA, body size as the covariate). $N_0$, sample size for control treatment group; $N_{\text{front}}$, sample size for treatment group that regenerated a front right leg; and $N_{\text{hind}}$, sample size for treatment group that regenerated a hind right leg.

N/A, not applicable.

Table 1. Effects of regeneration on front, mid, and hind legs on A. buprestoides

For both males and females, no resource allocation trade-offs were apparent with any of the other measured traits (antennae, eyes, defensive glands, cerci, female genital plates, or male testes) (Tables 2 and 3). This was true for the data analyses with both body size and body mass as the covariate, so only the ANCOVA results with body size as the covariate are reported (Table 3). In addition, there were no differences between the individuals that regenerated a front right leg and those that regenerated a hind right leg, so for simplicity with respect to nonleg trait-trade-offs, those individuals were pooled into one large "1 leg
regenerated” treatment group for the final analyses (Table 3).

Discussion

Results from this study showed three key results. First, regenerated legs were smaller than nonregenerated legs in both males and females, and although this may be accompanied by fitness costs, trade-offs as they were studied here focused on how the resources allocated to leg regeneration affected the growth of other traits. Second, females showed no trade-offs with any of the traits measured in this study, including body size. It is possible females show a trade-off between leg regeneration and fecundity (like another wingless phasmid, C. morosus), but fecundity was not measured in this study. And third, the resources allocated to regrow a leg in males did not come at the expense of any measured trait except body size; surprisingly, males that regenerated a leg were larger (but not heavier) than nonregenerating males. This effect of regeneration on male body size in this study is not intuitive and requires further investigation.

In addition to a general exploration into the effects of regeneration on A. buprestoides, our study design allowed us to test for two key factors that are important for questions regarding how trade-offs are manifested in arthropods. First, we looked for trade-offs with traits that directly overlapped with the regrowth of a leg (e.g., antennae, eyes, defensive glands, legs), as well as traits that only develop at later instars (e.g., testes, genital plates, and cerci). Thus, we were able to test for the occurrence of developmental resource allocation trade-offs between traits that grew either simultaneously with, or immediately after, leg regeneration. Second, we tested whether trade-offs were physically localized. In several species of Onthophagus dung beetles, for example, trait-trait trade-offs are most prevalent between the closest or most adjacent traits; horns that grow at the base of the head stunt the growth of the neighboring antennae, horns that grow at the back of the head stunt the nearby eyes, and horns that grow from the thorax stunt adjacent wings (Emlen 2001). As holometabolous developers, these trade-offs may persist because horns, antennae, eyes, and wings all go through the most growth after the animals have stopped feeding, and compete for stored resources in a closed system (Nijhout 1994, Emlen 2001).

Table 2. Average and standard deviation of measured traits in A. buprestoides

| Trait                      | Hind legs (length, cm) | Antennae (area, mm) | Eyes (area, mm) | Glands (area, mm) | Cerci (area, mm) | Genital plate (area, mm) | Testes (wt, µg) |
|----------------------------|------------------------|---------------------|----------------|------------------|------------------|-------------------------|----------------|
| N0                        | 2.21 ± 0.16            | 1.50 ± 0.07         | 0.43 ± 0.04    | 7.75 ± 1.51      | 1.27 ± 0.09      | N/A                     | 0.29 ± 0.08     |
| N1                        | 2.34 ± 0.13            | 1.56 ± 0.09         | 0.46 ± 0.06    | 7.54 ± 1.31      | 1.35 ± 0.09      | N/A                     | 0.33 ± 0.10     |
| N/A                       | 1.59 ± 0.10            | 0.47 ± 0.02         | 8.32 ± 2.27    | 1.33 ± 0.05      | N/A              | 0.22 ± 0.07             |
| N0                        | 3.04 ± 0.17            | 3.45 ± 0.13         | 1.72 ± 0.16    | 25.78 ± 5.13     | 1.35 ± 0.15      | 2.39 ± 0.20             | N/A            |
| N1                        | 3.15 ± 0.13            | 3.44 ± 0.14         | 1.70 ± 0.20    | 27.13 ± 6.14     | 1.38 ± 0.26      | 1.93 ± 0.25             | N/A            |
| N/A                       | 3.51 ± 0.17            | 1.56 ± 0.18         | 25.08 ± 6.00   | 1.42 ± 0.20      | 2.07 ± 0.19      | N/A                     |

Table 3. Effects of leg regeneration on other structures in A. buprestoides

| Trait                      | Antennae (length, in cm) | Eyes (length in cm) | Glands (length in cm) | Cerci (length in cm) | Genital plate (length in cm) | Testes (wt, µg) |
|----------------------------|--------------------------|---------------------|-----------------------|----------------------|-------------------------------|----------------|
| N0                        | 3.46 ± 0.19              | 0.213 ± 0.04        | 2.03 ± 0.13           | 1.52 ± 0.10          |                               |                |
| N1                        | 3.61 ± 0.19              | 0.220 ± 0.03        | N/A                   | 1.56 ± 0.08          |                               |                |
| N0                        | 3.57 ± 0.12              | 0.223 ± 0.03        | 2.11 ± 0.07           | 1.57 ± 0.07          |                               |                |
| N1                        | 6.00 ± 0.33              | 1.50 ± 0.37         | 2.83 ± 0.15           | 2.26 ± 0.12          |                               |                |
| N0                        | 6.11 ± 0.16              | 1.60 ± 0.51         | N/A                   | 2.33 ± 0.09          |                               |                |
| N1                        | 6.13 ± 0.27              | 1.76 ± 0.51         | 2.93 ± 0.14           | 2.33 ± 0.11          |                               |                |

N0, sample size for control treatment group; N1, sample size for treatment group that regenerated one leg (front and hind leg groups combined). N/A, not applicable.
Phasmids are incomplete metamorphic insects. For example, regenerating a front leg could have negatively impacted the nearby defensive glands and had no effect on the relatively distant testes. This study showed no evidence of localized trade-offs, but more research is needed into the hypotheses regarding the localization of trait-trait trade-offs in incompletely metamorphic insects.

We also hypothesized that the long life history of *A. buprestoides* may have alleviated trade-offs that were predicted to arise. Previous studies have also shown how the prevalence of resource allocation trade-offs can vary based on life history. In short-lived holometabolous caddisflies, rebuilding a nymphal case (an expensive silk structure produced by the larvae for protection from predators) comes at the expense of adult wing and thorax size (presumably an indicator of the muscles and lipids used to power flight, Stevens et al., 1999). Although long-lived caddisflies that must rebuild a case also develop smaller wings, their thorax weight is unaffected; they show a trade-off with abdomen weight, a predictor of the animal’s investment into reproduction (Stevens et al. 2000). Evolutionary theory suggests that short-lived organisms preserve fecundity, whereas long-lived organisms preserve muscles and fuel to power flight. Similarly, in vertebrates, short-lived lizards will not regenerate lost tails and instead use those resources for reproduction (e.g., they experience no resource allocation trade-offs due to regeneration; Dial and Fitzpatrick 1981). In contrast, some long-lived salamanders will abandon reproduction when faced with a lost tail, and invest all its energy into regeneration (e.g., the resources allocated to tail regrowth come at the direct expense of fecundity; Maiorana 1977). Much like caddisflies, evolutionary theory suggests that lifetime fecundity of a short-lived species is determined through a single breeding season; selection would favor investing into reproduction rather than regeneration, whereas long-lived species can afford to sacrifice one breeding season to allocate resources to tail regrowth (Maiorana 1977, Dial and Fitzpatrick 1981, Hill et al. 1988, Smith and Hines 1991). In phasmids, regenerating a lost appendage is not facultative; individuals always regenerate lost legs, no matter when the leg(s) were lost (T.L.M., unpublished data). Consequently, selection cannot currently act on phasmids as it apparently has in other taxa.

In conclusion, leg regeneration in *A. buprestoides* showed no apparent resource allocation trade-offs with respect to a suite of traits. These results may be explained by their long life history, the ad libitum food conditions, or perhaps that there are trade-offs that lie in unmeasured traits (e.g., fecundity). More work at the physiological and ecological level across a variety of species will hopefully elucidate the nature of acquisition-allocation trade-offs in phasmids, and how those trade-offs may have shaped their evolution.

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