

Tail loss compromises immunity in the many-lined skink, *Eutropis multifasciata*

Chi-Chien Kuo · Chiou-Ju Yao · Te-En Lin ·
Hsu-Che Liu · Yu-Cheng Hsu · Ming-Kun Hsieh ·
Wen-San Huang

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Abstract Tail autotomy incurs energetic costs, and thus, a trade-off in resource allocation may lead to compromised immunity in lizards. We tested the hypothesis that tailless lizards will favor constitutive innate immunity responses over an energetically costly inflammatory response. The influence of fasting and colorful ornamentation was also investigated. We experimentally induced tail autotomy in the lizard *Eutropis multifasciata* and found that inflammation was suppressed by tail loss, but not further affected by fasting; the suppressive effect of colorful ornamentation was manifested only in males, but not in females. Constitutive innate immunity was not affected by any of these factors. As expected, only costly inflammation was compromised, and a less expensive constitutive innate immunity might be favored as a competent first-line defense during energetically demanding periods. After considering conventional trade-

offs among tail regeneration and reproduction, further extending these studies to incorporate disease risk and how this influences escape responses to predators and future reproduction would make worthwhile studies.

Keywords Colorful ornamentation · Disease risks · Immunity · Lizards · Tail autotomy

Introduction

Tail autotomy is a widespread tactic among lizards (Arnold 1984). The wriggling of the voluntarily shed tails distracts predators, thus enhancing the survival of lizards. The autotomy site is readily healed and tails are mostly regenerated (Arnold 1984). Despite the benefits of predator evasion, tail autotomy also entails energetic costs, including loss of tail fat reserves and energy expenses for caudal regeneration (Bateman and Fleming 2009), which will in turn limit energy reserves for competing physiological functions. Indeed, tail loss has lowered reproductive output and overwinter survival in several lizard species (Bateman and Fleming 2009).

Because immune defense costs energy (Klasing 2004), it is likely that tail loss will also compromise immunity; this knowledge helps give insight into the evolution of caudal autotomy but is currently lacking. The complex and multi-layered vertebrate immune systems are generally divided into intricately interrelated innate and adaptive components: innate immune response stimulates adaptive immunity, which in turn reinforces innate immunity (Abbas and Lichtman 2003). The adaptive component can be further divided into T cell-mediated immunity (mediated by T lymphocytes) and humoral immunity (antibody, produced by B lymphocytes) and is distinctive for its high specificity for microbes and capability to mount stronger and more

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C.-C. Kuo · C.-J. Yao · W.-S. Huang
Department of Zoology, National Museum of Natural Science,
Taichung, Taiwan

T.-E. Lin
Department of Zoology, Endemic Species Research Institute,
Nantou, Taiwan

H.-C. Liu · M.-K. Hsieh (✉)
Graduate Institute of Microbiology and Public Health,
National Chung Hsing University, Taichung, Taiwan
e-mail: mhsieh@dragon.nchu.edu.tw

Y.-C. Hsu
Department of Natural Resources and Environmental Studies,
National Dong Hwa University, Hualien, Taiwan

W.-S. Huang (✉)
Department of Life Sciences, National Chung Hsing University,
Taichung, Taiwan
e-mail: wshuang@mail.nmns.edu.tw

effective immune responses against repeated infections of the same microbes. While adaptive immunity is induced by exposure to foreign substances and can recognize and eliminate specific antigens, innate immunity comprises constantly circulating but less specific cells (phagocytes and natural killer cells) and proteins (complementary system proteins and natural antibodies) capable of eliminating microbes well before the development of adaptive immunity. Once the epithelial barrier is penetrated by microbes, constitutive (continuously produced) cells and proteins of the innate system are rapidly activated, followed by induced inflammation reaction (Abbas and Lichtman 2003). Although inflammatory response helps recruit leucocytes and plasma proteins into the infected tissue and is efficient in eradicating microbes and containing the infection (Abbas and Lichtman 2003), inflammation, especially the acute phase response, incurs significant energetic costs despite not having a particularly high protection value (Klasing 2004). Therefore, we expect that costly inflammatory responses will be compromised once lizards lose their tails.

The many-lined skink *Eutropis multifasciata* (formerly *Mabuya multifasciata*) is a medium-sized (snout–vent length (SVL) ~100 mm), viviparous scincid lizard commonly found throughout southern Taiwan that has a high frequency of tail breaks (~57 %) (Sun et al. 2009). Some individuals of both sexes display a lateral bright yellow or orange stripe behind the axilla, but the function of such colorful ornamentation remains undetermined (Auffenberg and Auffenberg 1989). Until now, reptilian immune functions are known to be affected by a number of factors, including age, sex, reproduction, stress hormones (e.g., corticosterone), sex steroids (e.g., testosterone), temperature, season, food availability, life history, and parasite load (Mondal and Rai 2001; French and Moore 2008; French et al. 2008; Sparkman and Palacios 2009; Huyghe et al. 2010; Ruiz et al. 2010; Zimmerman et al. 2010), but how tail loss will influence immunity in lizards remains poorly investigated. In this study, the following were performed: (1) We tested the hypothesis that tail-shedding *E. multifasciata* will compromise induced inflammatory responses but preserve constitutive innate immunity as a competent first-line defense. (2) Furthermore, because food resources could influence immune functions in lizards (Ruiz et al. 2010) and tail loss may impair locomotor performance in *E. multifasciata* (Sun et al. 2009), which could reduce food intake, we tested whether fasting has a further suppressive effect on immunity of tailless individuals. (3) Lastly, immune responses could vary with sexual colorations (Kotiaho 2001), which has important implications for the maintenance and dynamics of different colorful morphs, but few studies have focused on lizards (but see Svensson et al. 2001). Because a display of colorful ornamentation may trade off with energetic investment in immunity (Verhulst et al. 1999; Kotiaho 2001), we hypothesized that *E. multifasciata* exhibiting colorful

stripes will have suppressed immunity compared with those not displaying colorful stripes. Similarly, we hypothesized that only inflammation, but not constitutive innate immunity, will be affected. In this study, tailless is defined as those individuals that have had their tails intentionally removed by the researchers. To our knowledge, this is the first study investigating immunity costs of tail loss in lizards.

Material and methods

Experimental tail removal

Our study was conducted from June to August 2011. A total of 86 *E. multifasciata* were caught by hand or in pitfall traps in southern Taiwan, measured for SVL and body mass, examined for the presence of an ornamental stripe behind the axilla, and sexed by gently everting the hemipenes, which were later confirmed by necropsy. Lizards were taken to the Natural Science Museum in central Taiwan, where the mean air temperature is similar to the capture site (<2 °C difference). We housed lizards individually in plastic containers (37.5×23×25 cm) containing shelter, located outdoors beneath a roof. Container locations were rotated frequently to ensure that each lizard was exposed to a similar thermal environment and had similar basking opportunities. Water was provided ad libitum, but food was restricted to three vitamin and calcium-dusted mealworms three times weekly to help reveal trade-offs among demanding physiological functions that unlimited food supplies can conceal (French et al. 2009). Animals were allowed to acclimate to captivity for at least 2 weeks before the experiment began (Jones and Bell 2004). Immature and gravid individuals, along with those undergoing vitellogenesis or shedding tails while captured or handled (instead of intentionally induced tail shedding by researchers) were excluded from further study or analysis. SVL of captured *E. multifasciata* ranged from 80.4 to 115.6 mm.

Reproductive capability is defined as the ability to form sperm (for males) or to become gravid or produce vitellogenic follicles >3.0 mm (for females) (Auffenberg and Auffenberg 1989); males with SVL > 79.0 mm and females with SVL > 86.6 mm are thus deemed as reproductively mature based on the minimum SVL of reproductive *E. multifasciata* in southern Taiwan (Lin, T.E., unpublished data). Gravidity and vitellogenesis were confirmed by observing females giving birth during the study (which were excluded from further immunity assays) or necropsy (if confirmed, these individuals were excluded from data analysis). *E. multifasciata* with signs of previous tail loss (but with largely regenerated tails upon capture) were included in the study, and statistical analysis (see below) confirms that the occurrence of previous tail loss does not significantly influence immunity. Individuals of similar sexes and

body mass (difference <5 g) were allocated evenly to one of three treatments: individuals whose tails were left intact with food provided (control group) and individuals whose tails were removed with or without food provided (treatment groups). We excluded a treatment involving lizards with intact tails and without food because our aim was to study the combined effect of tail loss and restrained foraging, instead of the latter alone. Caudal autotomy was induced by clipping the proximate tail (~3 cm from the vent, where tail autotomy occurs most frequently (Sun et al. 2009)) using binder clips. Individuals of the control group were also retrieved from their cage and handled, but the tails were left intact.

Immunity assays

We conducted constitutive innate immunity and inflammatory response assays 1 week after tail removal to minimize the influence of handling stress on these metrics. The inflammatory response assays were done first because lizards had to be sacrificed for blood collection during the constitutive innate immunity assay. We injected a 20- μ l solution containing 5 mg of phytohemagglutinin (PHA-P, Sigma, St. Louis) dissolved in 1 ml of phosphate-buffered saline (PBS) into one hind foot pad, and 20 μ l of PBS into the other hind foot pad. PHA is a mitogen common in immunity assays in vertebrates, including lizards (Calsbeek et al. 2008; Cox et al. 2010; Huyghe et al. 2010). Histological evidence shows that the PHA-induced swelling responses measured 24 h post-PHA-injection are more reflective of inflammatory potential than T cell-mediated immunity (Kennedy and Nager 2006; Martin et al. 2006; Vinkler et al. 2010). We therefore define PHA-induced swelling as an inflammatory response, as measured by the difference in the thickness of the two hind foot pads 24 h post-injection (mm), with each foot pad measured three times using digital calipers (to 0.01 mm) to obtain an average.

Two weeks later (when PHA-induced swelling was confirmed non-existent; see below), we bled the lizards to quantify constitutive innate immunity. Therefore, the fasted group was without food for 1 and 3 weeks before assaying PHA-induced swelling response and constitutive innate immunity, respectively. A vial containing 10 μ l of whole blood dissolved in 70 μ l of CO₂-independent media was added to a 25- μ l refreshed microbial solution containing about 250 colony forming units (CFU) of *Escherichia coli* (gram-negative, ATCC-25922) or *Staphylococcus aureus* (gram-positive, ATCC-29213), which was incubated at 32 °C (the preferred temperature for *E. multifasciata* (Ji et al. 2007)) for 2 h (*E. coli*) or 4 h (*S. aureus*). Both gram-negative and gram-positive bacteria were included because they measured different aspects of constitutive innate immunity (Liebl and Martin 2009). The concentration and reaction time were determined from preliminary tests of optimization between bacterial growth and antimicrobial capacity. Positive controls contained

80 μ l of CO₂-independent media mixed with a 25- μ l microbial solution, conducted at the beginning and end of each bout and following the same procedure. After incubation, the solution from each vial was pipetted onto two duplicate agar plates (each with 50 μ l), spread, and cultured at 37 °C for 12–16 h. CFU for each plate was then counted and averaged for two duplicates (four duplicates for positive controls). Constitutive innate immunity, represented by antimicrobial capacity, was calculated as $1 - (\text{CFU of individual lizard} / \text{CFU of positive control})$.

Statistical analyses

The effect of tail removal, sex, and stripe on each of three immune responses (PHA swelling and antimicrobial capacity against *E. coli* and *S. aureus*) was analyzed using separate ANCOVAs, with SVL as the covariate to control for potential influence of body size on reptilian immunity (Ujvari and Madsen 2006; Zimmerman et al. 2010); if significant, we used a Tukey's honestly significant difference (Tukey's HSD) test for post-hoc comparisons. Because normality and homogeneity of variance could not be achieved even after transformation, we used ranked input rather than raw data, making this a non-parametric analysis (Conover and Iman 1981). In addition, type III sum of squares ANCOVAs were implemented due to the unbalanced data structure and an existence of interaction among fixed effects (SAS Institute Inc. 2009). Previous tail loss (and all interactions) did not affect immunity (all $p > 0.05$) and was not included in further analysis. PHA-induced swelling was confirmed non-existent (see "Results") by comparing the difference in thickness of two hind foot pads 2 weeks post-PHA-injection with a paired *t* test. When comparing SVL and body mass between tail removal treatments or comparing SVL between striped and non-striped individuals, we used parametric ANOVAs or *t* tests or non-parametric Kruskal–Wallis or Mann–Whitney *U* tests, depending on whether assumptions could be fulfilled (as with the ANCOVAs). Mean values are followed by \pm SE, and raw data (instead of ranks in ANCOVAs) are shown for easy inspection. All procedures were implemented in JMP 8 (SAS Institute, North Carolina).

Results

A total of 70 *E. multifasciata* were included in this study. The thickness of two hind foot pads (injection of PHA and PBS for each foot pad, respectively) was similar 2 weeks after PHA injection (paired *t* test, $t = 1.11$, $df = 68$, $p > 0.05$). Experimentally removed tails weighed 12.2 ± 0.5 % (mean \pm SE) of the entire body mass ($n = 45$), and there was no difference in SVL and initial body mass among the three tail removal treatments (ANOVA, $F_{2,67} = 0.06$, 0.09, respectively; both $p > 0.05$). PHA-induced swelling was affected by

tail removal (ANCOVA, $F_{2,57}=10.20$, $p<0.001$), with tailed lizards having stronger swelling response (0.31 ± 0.03 mm, $n=25$) than tailless lizards, with (0.14 ± 0.03 mm, $n=21$) or without (0.20 ± 0.03 mm, $n=24$) food provided (Tukey's HSD, both $p<0.05$); however, there was no significant difference between the later two groups ($p>0.05$; Fig. 1a). Swelling response was not affected by the existence of a colorful stripe, sex, or SVL (all $p>0.05$; Table 1). However, there was a significant interaction among sex, stripe, and PHA swelling response ($F_{1,57}=4.05$, $p<0.05$; Fig. 1b): swelling was suppressed only in striped males (0.16 ± 0.02 mm, $n=37$; 0.32 ± 0.04 mm, $n=9$; for striped and non-striped, respectively; $p<0.05$), but not in striped females (0.28 ± 0.05 mm, $n=11$; 0.29 ± 0.06 mm, $n=13$; $p>0.05$). Although males with or without stripes were similar in SVL (with vs. without, 99.9 ± 1.2 vs. 104.3 ± 2.5 mm; t test, $t=1.59$, $df=44$, $p>0.05$), females with colorful stripes were larger than those without (with vs. without, 107.7 ± 1.2 vs. 98.7 ± 1.3 mm; $t=5.15$, $df=22$, $p<0.001$). Antimicrobial capacity against *E. coli* or *S. aureus* was unaffected by any of these factors (all $p>0.05$; Table 1).

Discussion

Our study shows that tail shedding compromised immunity in *E. multifasciata*, but immunity was similar in tailless

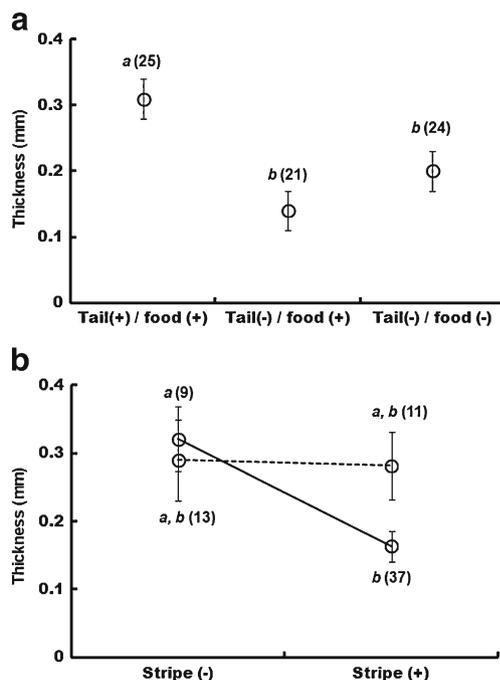


Fig. 1 The effects of (a) tail removal and food provisioning and (b) sex and ornamental stripes (solid line for males; dashed line for females) on inflammatory responses (represented by PHA swelling response, mean \pm SE) of *E. multifasciata*. Different letters dictate significant differences ($p<0.05$); sample size is shown in parentheses. Raw data are shown for easy interpretation

lizards irrespective of the level of food provisioning. In addition, having a colorful display stripe was associated with suppressive immunity in males, but not in females.

As expected, only the very costly inflammatory response, represented by PHA-induced swelling, but not the less expensive constitutive innate immunity, was compromised by tail loss. Our results agree with the hypothesis and other empirical studies that costly inflammation will be downregulated when individuals engage in demanding activities (Lee 2006). Possibly, maintaining a competent first-line constitutive innate immune defense benefits *E. multifasciata* by preventing the induction of more costly inflammation; simply having lower energy reserves could also compromise expensive immune functions.

Caudal autotomy affects *E. multifasciata* immunity, even when the removed tails only weighed about 10 % of body mass. This is not surprising given the reallocation of limited resources for caudal regeneration and the significant fat deposits in the tails of many lizard species, including *E. multifasciata* (Sun et al. 2009). A reduction in innate immunity has been observed in larvae of damselfly with lamellae autotomy (Slos et al. 2009). A lower tail regeneration rate in parasitized lizards compared with non-parasitized ones may also suggest a trade-off between caudal regeneration and immune defense against parasite infections (Oppliger and Clobert 1997). Nevertheless, we did not find a further suppressive effect of fasting on inflammatory response. This may be due to the already restricted food supply and the short fasting period (1 week before PHA injection): both can lead to a limited difference in food intake between the two tailless groups. Adding to this is that, unlike mammals or birds, reptiles do not maintain high body temperatures, and their physiological functions may thus be less compromised by scarce resources. Furthermore, lizards may save energy by reducing their activity when confronted with food shortages so that essential physiological functions, including immunity, can still be maintained. On the other hand, the aggregation of immune cells and proteins around the healing and regenerating tails could decrease their circulation around other body parts, leading to a reduced PHA swelling response in the foot pads of tailless lizards. Such trade-offs within the immune system, instead of among competing physiological functions, may also explain the insignificance of food intake in affecting PHA swelling response. This hypothesis, nevertheless, does not explain the similar antimicrobial capacity of blood in tailed lizards and the lizards that were still regenerating the removed tails, nor does it explain the reduced PHA swelling response in striped versus non-striped males (see below) irrespective of tail loss. Still, the reallocation of immune cells and proteins could occur in tail-regenerating lizards, and their merits in relieving competing physiological functions deserve further investigation.

Both sexes of *E. multifasciata*, especially males, regularly display a conspicuous lateral stripe, but its function

Table 1 Results of ANCOVAs (F values) for immune responses of *E. multifasciata* to tail removal, sex, and ornamental stripe, using SVL as the covariate

Effect	df	Inflammation (PHA swelling response)	Constitutive innate immunity (antimicrobial capacity)	
			<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Tail	2	10.20**	1.93	0.14
Sex	1	0.05	1.52	3.62
Stripe	1	2.32	3.17	0.66
SVL	1	0.43	0.22	0.40
Tail*sex	2	2.74	0.41	0.28
Tail*stripe	2	1.45	0.16	0.24
Sex*stripe	1	4.05*	0.13	2.14
Tail*sex*stripe	2	0.95	2.37	0.14

* $p < 0.05$ ** $p < 0.001$

remains unknown. In the Philippines, the occurrence of stripes in males generally synchronizes with enlarged testicles, suggesting a display of reproductive readiness (Auffenberg and Auffenberg 1989); however, a colorful stripe also exists in immature males in Taiwan (Lin, T.E., unpublished data). Indeed, SVL is similar between males with and without stripes. Whatever the role for stripe display, colorful ornamentation could reduce inflammatory responses in male *E. multifasciata* when food intake is limited. This could occur via an energetic trade-off between color deposition and immunity, or the immunosuppressive effect of testosterone, which stimulates coloration (Kotiaho 2001; Roberts et al. 2004; Cox et al. 2005). On the other hand, a colorful display stripe did not affect immunity in female *E. multifasciata*, and striped females had larger SVL than non-striped ones. Coloration in female lizards may signal reproductive receptivity or dominance (Calisi et al. 2008). Because reproductive females (including gravid females and those experiencing vitellogenesis) were excluded from this study, and the remaining non-reproductive females included both striped and non-striped ones, presence of a stripe does not seem indicative of reproductive stage (reproductive females also comprised both striped and non-striped ones; Kuo, C.C., unpublished data). Instead, a color display in female *E. multifasciata* may signal superior phenotypic quality, as striped individuals were larger than non-striped ones. Coloration was similarly associated with larger body size and better body condition in female striped plateau lizard (*Sceloporus virgatus*) (Weiss 2006). Likely, the larger body size and potentially higher dominance of striped *E. multifasciata* would enable those females to acquire more or better resources to offset the costly investment in stripe formation, thus resulting in a similar level of immunity as non-striped individuals.

Because immunity costs energy, there is likely a conflict in terms of longevity in allocating resources to caudal regeneration or immunity. Although tail regeneration can facilitate predator escape and increase longevity, impaired immunity may expose lizards to increased disease infection, plausibly reducing survival. After considering conventional trade-offs among tail regeneration and reproduction (Maiorana 1977), taking disease risk into account and how this affects predator escape and reproduction would make worthwhile studies.

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