Arthropod Structure & Development 59 (2020) 100995

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Prevalence of leg regeneration in damselflies reevaluated: A case study in Coenagrionidae

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ARTICLE INFO

Article history:
Received 15 June 2020
Accepted 27 August 2020
Available online 23 September 2020

Keywords:
Odonata
Morphology
Limb development
Autotomy

ABSTRACT

The leg regeneration capabilities of damselflies are understudied. Here we present the first data of regenerated limbs across a genus of damselfly based on adult specimens collected in the field to illustrate the prevalence of limb loss among nymphs. We show that this phenomenon is much more prevalent than previously thought, as 42 percent of individuals were found with regenerated limbs. Furthermore, we test for patterns within these data to begin to unravel the potential causes of limb loss in nymphal damselflies, showing that intrinsic factors such as sex and species cannot explain the patterns of limb loss pointing to environmental factors as the probable cause. We argue that Odonata limb regeneration provides a potentially unique perspective into the nymphal stage of these organisms.

1. Introduction

The phenomenon of structural regeneration, the act of regrowing damaged body parts (e.g., limbs), has been fascinating scientists for decades (Alvarado and Tsonis, 2006). From lizards to crabs, this ethereal ability has been viewed by many as the key to unlocking similar regeneration capabilities in our own species (Alvarado and Tsonis, 2006; Maginnis, 2006a). Arthropods, in particular, are capable of extensive structural regeneration (Maruzzo et al., 2005; Bely and Nyberg, 2010; Suzuki et al., 2019), but this can vary between groups. For example, regeneration in insects is different from that of other arthropods as successive molting is often required to complete the process, and adults therefore cannot regenerate limbs (Truby, 1983; Khan et al., 2016; Das, 2015; Suzuki et al., 2019). The absence of regeneration in adults makes it more difficult to study the causes and effects of limb loss in insects than in other groups where injuries are more easily studied, such as crustaceans in which adults continue to molt and regenerate throughout their life (Suzuki et al., 2019). There is a distinction between hemimetabolous insects whose regenerative capabilities seem very similar to that of other arthropods, such as crabs, and holometabolous insects whose regeneration is complicated by a pupal life stage required for complete metamorphosis (Das, 2015). In holometabolous insects, such as Coleoptera (Shah et al., 2011; Abdelwahab et al., 2018), Lepidoptera (Yang et al., 2016), and Diptera (Bosch et al., 2010), it is likely that imaginal cells or discs play an important role in their regenerative abilities (Lee et al., 2013; Das, 2015). In hemimetabolous insects, such as Ephemeroptera (Almudi et al., 2019), Odonata, Orthoptera (Yang et al., 2016), Phasmatodea (Maginnis, 2006b), Blattodea (Tan et al., 2013), and Hemiptera (Knobloch and Steel, 1988), there is a “breakage point” on the limb that results in the limb easily falling off when subject to pressure, a mechanism called autotomy (Maruzzo et al., 2005; Maginnis, 2006b). It has been suggested that the ability to break off limbs and regenerate them allows these organisms to better survive predators and intraspecific aggressors (Bely and Nyberg, 2010). Furthermore, this common breaking point could enhance healing and prevent signaling potential predators to weakened individuals (Matsuoka and Ishihara, 2010; Maruzzo et al., 2005).

Odonates, a hemimetabolous order encompassing dragonflies and damselflies, have effective regeneration abilities with regard to both, legs and caudal lamellae (Maruzzo et al., 2005; Stoks and De Block, 2007). The loss and regeneration of caudal lamellae is relatively well-studied in damselfly nymphs and is largely attributed to interactions with other organisms such as conspecifics and predators (Robinson et al., 1991; Black et al., 2019). The loss of lamellae is also considered to occur frequently with natural populations having anywhere from 50% to 90% of individuals with lost lamellae (Robinson et al., 1991; Stoks, 1998; Stoks and De Block 2000). Leg

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https://doi.org/10.1016/j.asd.2020.100995

regeneration, on the other hand, is poorly studied perhaps due to some evidence that it occurs infrequently, with nymphs largely relying on caudal lamellae autotomy rather than leg autotomy to escape predation (Robinson et al., 1991; Stoks and De Block, 2007). Perhaps owing to this assumption, little work on the leg regeneration capabilities of the order has been done beyond that completed in the early twentieth century. Furthermore, the underlying causes and impacts of limb regeneration on individuals are unknown. Although the majority of the research focuses on the caudal lamellae and limbs of damselflies (suborder Zygoptera), there is some evidence that similar regeneration occurs in dragonflies (suborder Anisoptera) (Przibram, 1909).

One of the earliest experiments was that of Child and Young (1903) who performed a series of experiments on damselfly nymphs (Agrionidae) to determine the effects of certain segments of the legs being removed. They provided an in-depth study of what the regenerated limb looked like after being removed from each section of the leg, as well as what they believed to be the breakage plane on odonates, between the femur and trochanter (Child and Young, 1903). They found that regeneration resulted in different levels of a “normal” leg depending on where the leg was cut. An attempt was made to summarize their findings (Table 1), however, it should be noted that there was variability within each of these scenarios and we only portray the most common results. Furthermore, depending on the age of the nymph, the time left during development that the leg had to regenerate was also a factor in some of these variable results. For example, when segments of the tarsus were removed from nymphs that were 27 days from their next molt, they had more developed claws and spines when compared to nymphs 18 days from their next molt (Child and Young, 1903). Furthermore, in conjunction with other work, Child and Young (1903) noted that when the leg was broken off at the articulation point between the femur and trochanter, there was less bleeding making this point a seemingly adaptive place to break off. Legs that regenerated at this breaking point, as well at the less-adaptive trochanter, form highly uneven non-articulated claws that appear as a single tarsal claw, as opposed to a normally formed pair of claws (Fig. 1) (Parvin and Cook, 1968; Forbes and Baker, 1989; McPeek, 1995). It appears that regenerated legs never regain a normal tarsal claw, even when the leg is broken off very early in nymph development (Forbes and Baker, 1989). This unusual tarsal claw led some researchers, without the background knowledge on regeneration, to wrongly hypothesize that the presence of abnormal tarsi in 35 percent of their study population was due to some sort of genetic mutation (Hilton, 1985). The idea that these limbs may represent genetic abnormalities was quickly refuted by Forbes and Baker (1989), however, it further highlights the limited scope of literature on this topic.

The unusual tarsal claw resulting from regenerated limbs broken off at this articulation point provides a unique opportunity to study the prevalence and possible factors affecting limb loss in nymphs within this order. This observable physical abnormality persisting through the often more readily collected and effectively preserved adult stage allows the investigation into the prevalence and patterns associated with nymphal leg loss with a much larger dataset. Prior work investigating this phenomenon has looked at how often nymphs of different Coenagrionidae species are wounded, finding a correlation between wound frequency and nymph dispersal rather than density as the authors had hypothesized (Baker and Dixon, 1986). More recently, some researchers have suggested that this ability should be utilized in non-lethal DNA sampling techniques within this order (Monroe et al., 2010; Ožana et al., 2020). However, more work needs to be done on the best method to cut the limb as well as the energetic and fitness costs that limb loss has on an individual before this method should be fully incorporated into conservation plans, especially when working with threatened species. Overall, the possible insights that limb regeneration may provide into different aspects of odonate ecology and behavior have yet to be fully employed.

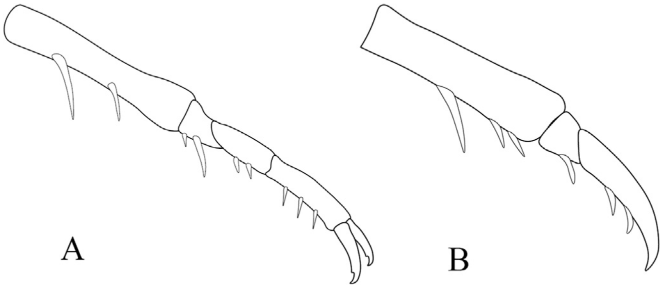

Fig. 1. A. Normal leg of damselfly; B. Regenerated leg of damselfly.

Vanuatubasis Ober and Staniczeck, are a group of endemic damselflies found on the islands of Vanuatu (Ober and Staniczek, 2009). There are currently only three species assigned to this genus; however, recent fieldwork in the region has resulted in the discovery of several undescribed species, each endemic to a single island in the archipelago (Marinov et al., 2019). While working on the taxonomy of this genus we noticed that many of the specimens had the abnormal tarsal claw described and illustrated by Forbes and Baker (1989). Further, the abnormal claw was found more often than expected based on the literature available. Here we present the first data looking at the prevalence of limb regeneration across a genus of damselfly. We attempt to both, rule out intrinsic factors related to leg loss, such as sex and species, and to provide insight into just how prevalent loss and regeneration of legs can be in odonate nymphs. Specifically, we look at the pervasiveness of limb loss between sexes and look at patterns of regeneration between islands (here used as a proxy for species due to the high levels of endemism) and test whether the loss of a specific pair of legs is correlated with either of these two factors.

2. Material and methods

We examined 202 *Vanuatubasis* specimens collected using aerial nets during the months of April, May and June across several field seasons spanning 2017–2019. Specimens were first sorted into six

Table 1
Summary of Child and Young (1903) regeneration study.

Cut at base of:	Trochanter	Femur*	Tibia	Tarsi	Tarsal Claw
Resulting form:	Similar to femur, but also appearing extremely wrinkled and deformed after first molt.	Reduced tarsi (two segmented), undeveloped claws (non-articulated and severely uneven —appear as single claw)	Reduced tarsi (two segmented), undeveloped claws (often uneven claws —usually two distinct claws)	Usually normal with possible tarsal reductions, largely dependent on time to molt	Normal, sometimes claw is smaller

*Articulation point.

island groups using both Leica GZ6 and Olympus SZ51 stereo microscopes. Only specimens with at least five visible legs were included in the analysis. For each specimen we recorded the number and location of the abnormal tarsal claws (appearing as a single claw), indicating that the leg had likely been regenerated from the articulation point, as well as the sex of the specimen. Structural abnormalities visible in the adult stage of these damselflies have been shown to be a direct result of a partial leg loss event during the nymphal stage of the organism, the most natural being a loss at the articulation point (Child and Young, 1903). This observation allows the use of data gathered from other odonate groups to investigate patterns associated with limb regeneration.

Statistical analyses were performed in SPSS Data Analysis Software (SPSS, Chicago, IL) to test for correlations between limb regeneration and the pair of legs, sex of specimens, and species of *Vanuatubasis*. Specifically, a one-way ANOVA was performed to test between sex or species, and the presence of abnormalities. Additionally, a MANOVA with a Wilks-Lambda Multivariate test was used to test whether sex or species was correlated with the presence of the abnormality on a specific pair of legs.

Imaging of specimens was done using a Vision Digital passport imaging system and then stacked using Zerene (Zerene Systems LLC, Richland, WA). Line drawings were produced in Adobe Illustrator v.22.1. Additional figures were generated in Microsoft Excel v.16.36. Specimens used in this study are housed at the Monte L. Bean Museum, Provo, Utah, USA.

3. Results

Of the 202 specimens examined across six islands, there were a total of 85 individuals (42%) with a leg matching that of Fig. 1B, indicating that regeneration had previously occurred from the articulation point (Table 2). In *Vanuatubasis* this regenerated limb has dense, small spines along the arch of the apical tarsal segment and ends in a sclerotized point (Fig. 1B). Of these 85 individuals, there were a total of 109 abnormalities. These were most commonly observed on the metathoracic legs with 41%, followed by the prothoracic legs with 32%, ending with the least frequent number of occurrences on the mesothoracic leg with 27% (Fig. 2). Across the specimens with confirmed presence of abnormalities, the majority only had one regenerated leg (76%), a further 19% had two regenerated legs, the final 5% had three confirmed regenerated legs (Fig. 3). No specimen examined had more than three regenerated legs. The island of Aneityum had the highest rate of regenerated legs at 75%, however, also the fewest number of specimens examined ($n = 4$). Efate had the lowest observed rate with 27% of legs having been regenerated. Proportions of specimens with regenerated limbs are given for each island in Table 2. Overall, island (here used as a proxy for species) was not recovered as a significant predictor of the presence of abnormalities (p-value $= 0.756$), nor the presence of abnormalities on a specific pair of legs (p-values $= 0.945$). When comparing between sexes, we found 29 females and 56 males with abnormalities. The regenerated legs were slightly more common in females (46%) than in males (40%) (Fig. 4). However, sex was not recovered as a significant predictor of overall presence of an abnormality (p-value $= 0.369$), nor abnormalities present on a specific leg (p-values $= 0.90$).

Table 2
Percent of specimens examined with regenerated legs listed by island.

Island	# Examined	% Abnormal
Maewo	29	45
Pentecost	54	44
Espiritu Santo	50	40
Malekula	39	46
Efate	26	27
Aneityum	4	75
Total	**202**	**42**

Total counts are bolded.

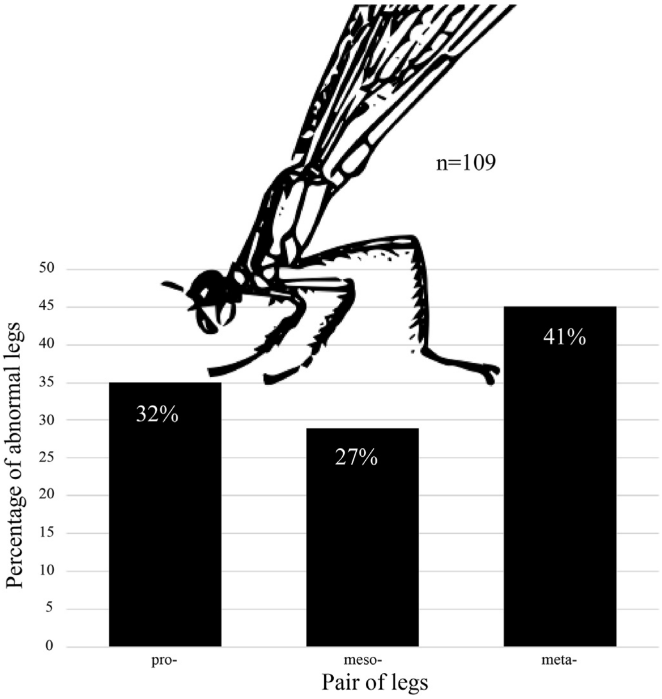

Fig. 2. Proportion of abnormality on each pair of legs.

4. Discussion

Our results show that leg loss and regeneration in odonate nymphs likely is significantly more widespread than has been assumed in the past, due to the observed prevalence among *Vanuatubasis*. The distinctly regenerated leg with a single tarsal claw and the reduced number of tarsal segments in the adults make the study of regeneration in damselfly nymphs easily observable. Our estimate most closely matches that of Hilton (1985) who found it in 35% of a single population of *Amphiagrion* Selys, believing it to be a "genetic abnormality". Robinson et al. (1991) found that leg

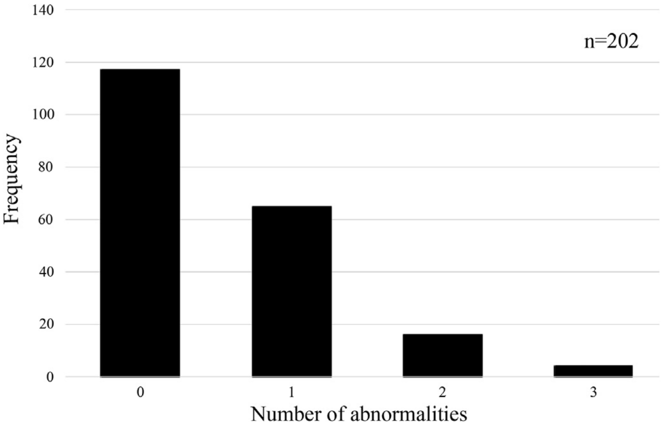

Fig. 3. Number of abnormalities on each individual examined.

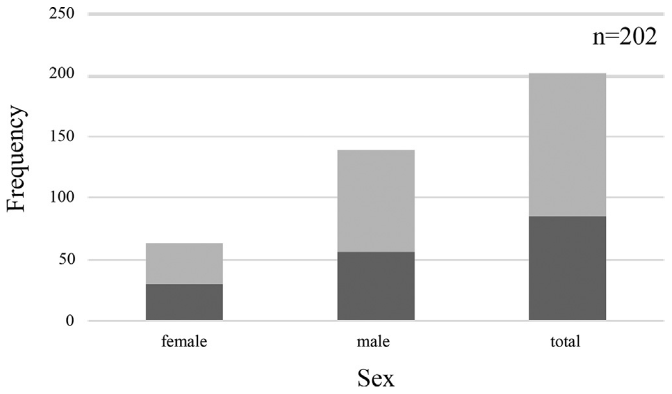

Fig. 4. Frequency of legs examined by sex. Dark grey represents the proportion of legs examined that were regenerated, light grey is the proportion of legs examined that were normal.

damage in nymphs occurred in their study populations at a range from 0 to 11%. This study led to Stoks and De Block (2007) to conclude that the phenomenon is likely rare in Odonata. These differences between what we observe and what other studies report may also be attributed to different microhabitats, predator regimes, behaviors and/or overall morphologies between different species of damselfly. For example, our results come from Coenagrionidae, a relatively robust nymph when compared to other odonate nymphs such as Lestidae. Due to the few studies looking at leg regeneration in other groups of Odonata, specifically in natural populations, it is difficult to assess the extent of structural regeneration across the group. The much higher regeneration rate of 42%, observed by us, demonstrates that there is considerable variability in the prevalence of leg loss and subsequent regeneration in damselflies and may point to specific environmental factors that require more investigation.

Our results indicate that intrinsic factors, such the sex of the individual or the species (islands used as a proxy for species), were not significantly affecting leg regeneration in nymphs. Furthermore, the loss of a specific pair of legs when tested with regard to both these variables was also not significant (Table 3). In ruling out two of the arguably most influential intrinsic factors that could lead to limb loss, we must instead consider the extrinsic environmental factors influencing nymphal limb loss and regeneration. Likely factors affecting nymphal limb loss, that should also be investigated, include stream flow rate, substrate, and number and density of predators. The observation that *Vanuatubasis* adults were primarily found on fast flowing, rocky bottomed streams (Fig. 5) lends credence to the idea that microhabitat condition could be a possible factor for limb loss in damselfly nymphs. Adult and nymphal damselflies use their legs for different functions further highlighting this division between life stages. In adults, legs are used largely for perching and prey capture, while nymphs use their legs for locomotion, burrowing, and support (Leipelt et al., 2010). It is conceivable that a damselfly nymph could suffer partial loss of a limb while grasping the rocky substrate in fast flowing conditions, particularly in areas with highly variable amounts of precipitation (e.g., Vanuatu). This idea is supported by the previously discussed frequency of the trauma occurring at the “breaking point” (Maruzzo et al., 2005). Predation as a possible driver still warrants investigation, as the regeneration events were most frequent on the metathoracic legs, which arguably would be the most commonly attacked pair of limbs at an individual attempt to escape. This hypothesis would align most closely with previous work done on the loss of caudal lamellae, as well as with observations that most predator attacks directed at other parts of the nymphs' bodies often end up in attacking the posterior end of the organism resulting in lamellae loss (Stoks and De Block 2000, 2007). However, the higher frequency of metathoracic leg regeneration could simply be a result of these legs being the longest pair of the three, giving it more opportunity to be damaged. Our dataset does include a large number of losses of the more anterior pairs of legs, shedding some doubt onto these explanations. One acute factor that could impact these patterns specifically in the South Pacific are tropical cyclones common from the months of November to April (Granderson, 2017). These storms release huge volumes of water into the watersheds on the islands of Vanuatu and cause rapid and extreme changes to the microhabitats where *Vanuatubasis* develops. All of the adults included in this study were collected in May or June, meaning many individuals were likely nymphs during the previous cyclone season. The increasing human impact on streams within Vanuatu is a convoluting factor, as islands with higher human population may have higher limb loss in *Vanuatubasis* due to stream disturbance; however, they may also provide a more stable environment due to increased water diversion and dams that reduce the chance of flooding. Our results show that the two most populated islands, Efate and Santo (see Table 2 in Hadden et al., 2020), had the lowest number of leg abnormalities with 27% and 40%, respectively (Table 2), which suggests that periodic flooding of streams in Vanuatu may play an important role in the limb loss in this genus.

Table 3
Results from statistical tests assessing the significance of sex, island, and sex and island on the prevalence of leg regeneration in *Vanuatubasis*.

Effect	F	df	*p*-value
Wilks' Lambda Multivariate Test			
Island	0.962	15	0.945
Sex	2.193	3	0.90
Island*Sex	1.740	12	0.56
Tests of Between−Subjects Effects			
Sex	0.811	1	0.369
Island	0.527	5	0.756
Sex*Island	1.544	4	0.191

Interesting further work could investigate the prevalence of limb loss and subsequent regeneration in other groups of odonates also found on these islands in similar microhabitats in order to test the impact of these specific environments. Related studies could also investigate if other genera of Coenagrionidae on the islands (e.g., *Ischnura* Charpentier or *Pseudagrion* Selys) that are more widespread have a lower abundance of regenerated legs than *Vanuatubasis*. One interesting study group might be exploring the limb-loss among species of *Indolestes* that often inhabit the same or very similar streams as *Vanuatubasis*. Ultimately, manipulated experiments rearing *Vanuatubasis* with varying types of substrates and flow rates as well as different predator regimes are needed to elucidate the extent to which each of these variables result in leg loss.

Regeneration in insects is distinct from that of other arthropods, as it only provides insight into the loss of limbs during the immature stage, as regeneration does not occur during the adult stage (Das, 2015). Here, we show that limb regeneration within the order Odonata can be more common than previously understood (e.g,. 42 percent), which further highlights how overlooked this

Fig. 5. Habitat of *Vanuatubasis* in Malekula, Vanuatu. A. Litslits, Malekula, Vanuatu; B. Amokh, Malekula, Vanuatu. (Photo credit: Colin Jensen).

phenomenon has been in the past. Our research provides a significant addition to damselfly regeneration research and hopefully spurs further investigation across the lineage. These data have the potential to provide a unique lens with which we can reconstruct portions of the natural history of damselflies that might otherwise be inaccessible due to the limitations of their aquatic lifestyle.

Author statement

NAS: Conceptualization, Data Curation, Writing-Original Draft, Writing-Review and Editing, Validation, Visualization. **GSP:** Conceptualization, Data Curation, Formal Analysis, Writing-Original Draft, Writing-Review and Editing, Validation, Visualization. **SMB:** Resources, Writing-Review and Editing, Validation, Funding Acquisition.

Declaration of competing interest

None declared.

Acknowledgements

The authors would like to thank Donna Kalfatakmoli for helping to secure collecting permits for Vanuatu. We also appreciate field assistance provided by Brigham Young University undergraduates. The authors also appreciate advice on statistical analyses from Alexandra Duffy and the use of images taken by Colin Jensen. Lastly, the authors thank the reviewers and editorial staff that greatly improved this work. The study was funded by Brigham Young University, and NSF DEB-1265714.

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