Article

Walking leg regeneration observed in three families and four species of antarctic sea spiders

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Received 1 September 2019; *Accepted 27 September 2019*; *Published 1 December 2019*

Abstract
Observations of wound healing and regeneration of walking legs in specimens of *Nymphon australe* Hodgson, 1902, *Nymphon charcoti* Bouvier, 1911, *Colssendeis tortipalpus* Gordon, 1932 and *Pentapycnon charcoti* Bouvier 1910 archived in the U. S. National Museum of Natural History collections is reported. One hundred and ninety-four specimens of *N. australe* were analyzed for evidence of regeneration. Blastema formation and or regenerated limbs of reduced size were found in 64 individuals, 38%. Forty-four specimens of *N. charcoti* were analyzed for evidence of regeneration. A blastema and/or regenerated limbs of reduced size were found in 12 individuals, 27%. Fifteen specimens of *Colssendeis tortipalpus* were analyzed for evidence of regeneration. Five individuals, 33%, had either a fully regenerated walking leg of reduced size or a blastema was present. Ten individuals of *Pentapycnon charcoti* were analyzed for evidence of regeneration. None of these animals showed signs of regeneration. The Preferred Breaking Point (PBP), position of autotomy, was between coxa 1 and coxa 2. Regenerated limbs having all segments, but of reduced size were found in 5 specimens. In some cases, more than one walking legs had been regenerated or were in the process of regenerating as indicated by the presence of a blastema. A blastema formed on the end of the first segment of the chelophore was observed in a single specimen of *N. australe*. A blastema which was formed at the end of the second segment of the ovigerous appendage of *C. tortipalpus* was also observed.

Keywords *Nymphon australe*; *Nymphon charcoti*; *Colssendeis tortipalpus*; *Pentapycnon charcoti*; regeneration; Preferred Breaking Point.

Arthropods
ISSN 2224-4255
URL: http://www.iaees.org/publications/journals/arthropods/online-version.asp
RSS: http://www.iaees.org/publications/journals/arthropods/rss.xml
E-mail: arthropods@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

The members of the phylum Arthropoda are capable of limb regeneration including eye stalks in crustaceans after autotomizing the lost appendage (Bely and Nyberg, 2009; Flemming et al., 2007; Maruzzo, et al., 2005; Maruzzo et al., 2013). They cannot regenerate the whole body or the core or body axis (Bely and Nyberg, 2009). Regeneration has been described in members of the class Pycnogonida, order Pantopoda from the later part of the nineteenth century (Dohrn, 1881; Gaubert, 1892; Morgan, 1901; Loeb, 1905). The abnormal regeneration of the posterior portion of the trunk with a limb-like structure was described for *Phoxichilidium femoratum* (Rathke, 1799) [as *Phoxichilidium maxillare* Stimpson, 1853] by Loeb (1905). The regeneration of walking legs was reported by Dohrn (1881) and Gaubert (1892). More recent work has shown that immature stages of pycnogonids can regenerate their walking legs, but not mature adults that have lost the ability to molt (Bely and Nyberg, 2009). This regeneration includes the cuticle, digestive tract and in some cases the gonads (Fage, 1949; Hefler and Schottke, 1935). Other than review articles surveying the regenerative process in arthropods the subject has received little attention in recent years (Maruzzo et al., 2005; Maruzzo and Bortolin, 2013).

Needham (1952) recognized two basic types of regeneration, epimorphosis and morphallaxis. Morphallaxis does not involve the proliferation of new cells; rather it results from the regeneration of the missing structures by the remodeling of existing cells. In morphallaxis, the remaining portion of the body is reorganized to restore the whole form. In epimorphosis, the former organ or limb is replaced by the direct development in situ of the portion of the animal which has been lost. Epimorphosis is typical of limb regeneration in arthropods. Epimorphacic regeneration requires cell proliferation and may be divided into non-blastemal and blastemal regeneration. Non-blastemal regeneration results from the trans-differentiation of the remaining tissue into the missing organ with limited proliferation of the surviving cells. Blastemal regeneration involves the formation of a specialized mass of cells known as a blastema. The latter is the type of regeneration found in the Arthropoda including the Pycnogonida (Mitić et al., 2010).

In the process of epimorphic regeneration Needham (1952) identified two main phases, regressive and progressive. The regressive phase included: Wound closure; Demolition of damaged cells and defense against foreign organisms and chemicals and the dedifferentiation of cells to provide new tissues for the progressive or repair phase. The progressive phase is divided into the formation of the blastemal, the growth of the blastema or regeneration bud and the differentiation of the young regenerate (Needham, 1952).

When a portion of a limb is lost or damaged it may be shed by the process of autotomy. In this process muscles constrict severing the portion of the limb distal to the Preferred Breaking Point (PBP) and sealing the end of the limb (Fleming et al., 2007). This process minimizes the damage to the remaining body of the animal and may also facilitate locomotion in the case of walking legs (Gross, 1969; Maginnis, 2006). In pycnogonids autotomy may also occur without subsequent regeneration as in the case of the shedding of cheliphores (Bain, 2003). The second and third larval appendages are lost in some species of pycnogonids only to be regenerated as palps and ovigerous appendages in the adults (Maruzzo and Bortolin, 2013).

Regeneration in arthropods can only occur in conjunction with molting and is subject to endocrinological controls. These are natural results of the existence of the chitinous exoskeleton. Limbs may be autotomized at specific joints, a process which facilitates wound healing and future regeneration (Gross, 1969). The process of molting is stimulated to occur by the presence of ecdysones in the blood of the animal (Krishnakumaran and Schniderman, 1970).

2 Materials and Methods

Archived specimens of *Nymphon australe* Hodgson, *Nymphon charcoti* Bouvier, *Colssendeis tortipalpus* and *Pentapycnon charcoti* Bouvier 1910 from the U. S. National Museum of Natural History were examined in this study for evidence of limb regeneration. Eight populations of Pycnogonida from the Antarctic Seas were analyzed in this study. Population A, USNM 1277471 including 29 specimens of *N. australe*. Population B including 77 specimens of *N. australe* collected at 76^0 $0.2^{'}$ S. 179^0 $52.1^{'}$ W. Population C including 45 specimens of *N. australe* collected at 66^0 17' 42'' S. 110^0 32' 03''E. Population D including 21 specimens of *N. australe* collected at 66^0 15' 24'' S. 110^0 28' 40'' W. Population E including 23 specimens of *N. australe*, collected at 73^o 49' S. 178^0 28' 13'' W. One population of *N. charcoti* including 44 specimens collected at 62^0 36.0' S. $64^0$14'30'' W. One population of *C. tortipalpus* including 15 specimens, collected from 61^o 18' S. 56^o 09' W to 61^0 20' S 56^o 10' W. including 10 specimens of *P. charcoti* each from a different location in the Antarctic seas.

Because limbs of pycnogonids can become broken while being collected, preserved or subsequently being handled, a set of criteria was established for defining when regeneration had occurred or begun. The first step in regeneration is the formation of a blastema at the site of limb severance. Like many arthropods, pycnogonids have the ability to autotomize a damaged limb. This process of severing a limb in response to an external stimuli is a defense mechanism from attack by a predator or injury. This occurs at a joint between two segments termed the Preferred Breaking Point (PBP) (Fleming et al., 2007). Regenerated limbs are physically smaller, i.e. they are shorter and thinner than mature un-regenerated limbs on the same animal at the first molt following regeneration. The presence of such a reduced limb was taken as evidence of regeneration. Lost limbs, without the presence of a blastema were considered to be limbs lost in processing of the samples. These lost limbs were not counted as having been limbs in the process of regeneration. In some cases this may have resulted in an under counting of regeneration events.

3 Results

3.1 *Nymphon australe* Hodgson

Population A: Ten adult and eight juveniles showed no signs of regeneration, limb loss or wound healing. One adult showed the results of regeneration producing smaller right walking legs 2 and 3 much thinner but the same length as normal walking legs. Two mature specimens showed early stages of regeneration in the form of a blastema on the 3^{rd} and 4^{th} right walking legs on one individual and a second individual with a blastema on the left 1^{st} and 2^{nd} walking legs. One specimen had blastemas on the 1^{st} and 4^{th} right walking legs. One specimen had blastemas on the 2^{nd} and 3^{rd} left walking legs plus the 4^{th} right walking leg. One specimen had a blastema on the 1^{st} and 2^{nd} right walking legs. One specimen had blastemas on the 1^{st} and 2^{nd} left walking legs. One had blastemas on the 2^{nd} and 4^{th} left plus the 3^{rd}right walking legs. One specimen had blastemas on the 2^{nd} and 4^{th} right walking legs. Two juveniles had lost limbs at the joint between the first and second coxa without a blastema, right 3^{rd} walking leg and 4^{th} left walking leg respectively. Unless otherwise noted the breaking point is between coxa 1 and coxa 2 (See Fig. 1).

Population B: Thirty seven individuals or 48% had missing walking legs which had formed a blastema and two individuals with regenerated walking legs, one with the right second and third walking legs present in a reduced size and one individual with the first left walking leg and second right walking leg present in a reduced size (See Fig. 2). One juvenile had blastemas on the 2^{nd} and 3^{rd} right walking legs. One juvenile had a blastema on the 3^{rd} left walking leg. Two juvenile specimens had blastema(s) on the 4^{th} left walking leg. Three juveniles specimens had a blastema on the 3^{rd} right walking leg. One juvenile specimen had blastemas on the 2^{nd}, 3^{rd} and left 4^{th} right walking legs. One juvenile specimens had blastemas on the 3^{rd} and left 4^{th}right walking legs.

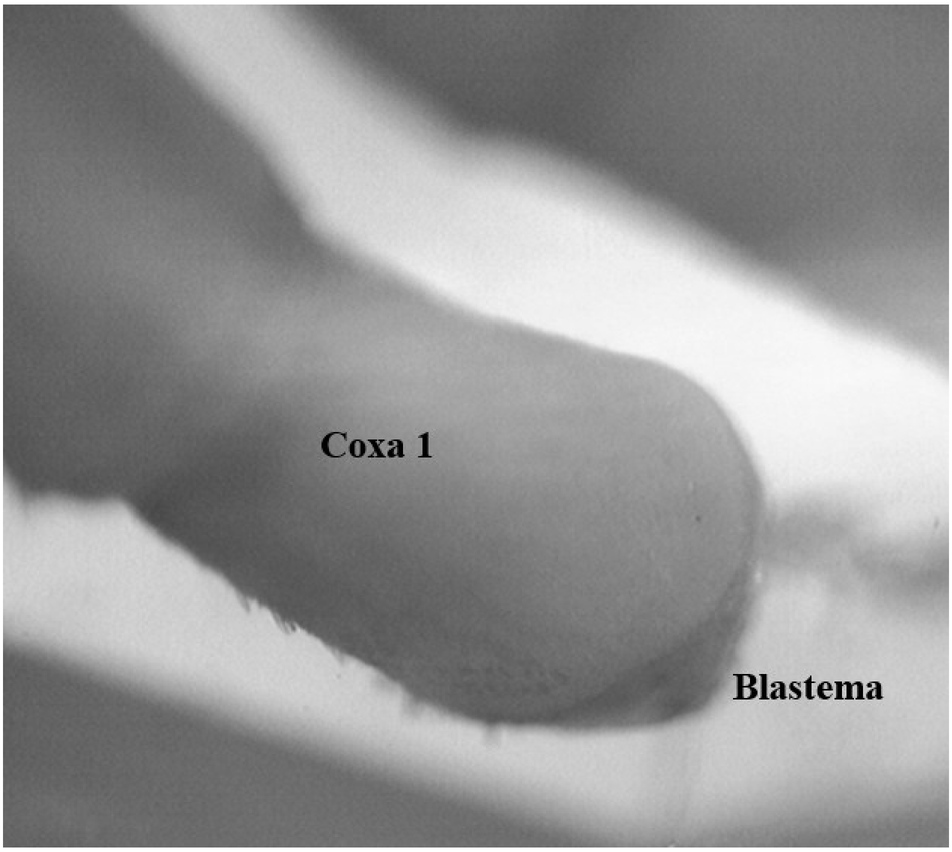

Fig. 1 The early stages of wound healing and regeneration of one of the walking legs of *N. australe* showing coca 1 and the blastema at the distal end of the autotomized limb.

Fig. 2 A ventral view of *N. australe* with a regenerated left first walking leg. This limb is shorter and thinner than the other walking legs.

Population C: Twenty-seven adults and six juveniles showed no signs of regeneration, limb loss or wound healing. One juvenile had a regenerated left first walking leg of reduced size. One juvenile had lost the second and third segments of the left chelophore. A blastema had formed at the distal end of the basal segment. One juvenile had a blastema on the left ovigerous appendage. One juvenile had a blastema on the 1st right walking leg. One juvenile specimen had blastemas on the 2nd left and right walking legs. Seven juvenile specimens had blastemas on two or more walking legs. One juvenile specimen without wound healing had a break at coxa 1/coxa 2.

Population D: Fifteen of the 21 adults showed no signs of limb regeneration. Six juveniles had initiated walking leg regeneration with the formation of a blastema. One specimen had a blastema on the 2nd right walking leg. One specimen had a blastema on the 2nd left walking leg. One specimen had blastemas on the 1st and 2nd right walking legs. One specimen had blastemas on the 3rd right and 4th left walking legs. One specimen had blastemas on the 1st2nd and 3rd walking legs.

Population E: Thirteen adult animals showed no signs of regeneration. Ten juveniles had lost and regenerated one or more walking legs and/or initiated regeneration by the formation of a blastema. Two specimens had blastemas on the 3rd and 4th left walking leg. One specimen had a blastema on the 4thright walking leg and a fully regenerated 4th left walking leg. Two specimens had blastemas on the 4th left walking leg. One specimen had a blastema on the 2nd left walking leg and fully regenerated 1st and 4th right walking legs. One specimen had a blastema on the first segment of the left chelophore and a reduced size regenerated left first walking leg. One specimen had a blastema on the on the 3rd left walking leg. One specimen had a blastema on the 1st right walking leg. One specimen had a blastema on the on the 3rd right walking leg (See Table 1).

Table 1 Summarizing the results for *N. australe.* The 193 missing walking legs all appeared to have been autotomized at the junction of coxa 1 and coxa 2.

Left legs	Number of limbs with a blastema	Right Legs	Number of limbs with a blastema
(I)	25	(I)	16
(II)	26	(II)	17
(III)	26	(III)	34
(IV)	25	(IV)	24

3.2 *Nymphon charcoti* Bouvier 1911

Thirty-two of the 44 specimens, 76%, were adult animals showing no signs of regeneration. Two juvenile animals had fully regenerated but smaller walking legs one with the first right walking leg present as a smaller limb and one with the second left walking leg present as a smaller leg. Ten juvenile animals, 24%, had lost one or more walking legs and had formed a blastema as the first stage of regenerating the lost limb. One specimen had a blastema on the right 2nd and 3rd walking legs. One specimen had a blastema on the 3rd left walking leg. One specimen had a blastema on the 4th left walking. One specimen had a blastema on the 3rd right walking leg. One specimen had blastemas on the 2nd, 3rd and 4th right walking legs. One specimen had a blastema on the 3rd left walking leg. One specimen had a blastema on the right 3rd walking leg. One specimen had a blastema on the 4th left walking leg. One had a blastema on the 3rd left walking leg. One specimen had blastemas on the 3rd and 4th right walking legs (See Table 2).

3.3 *Colssendeis tortipalpus*

Ten of the fifteen specimens of this species were adults and showed no signs of walking leg regeneration. One adult, however had lost the segments distal to the third segment of the right ovigerous appendage and a blastema had formed at the distal end of that segment (See Fig. 3). One of the juvenile specimens had a fully regenerated, but small, 4thleft walking leg. One juvenile had a blastema at the distal end of coxa 1 of the 1st right walking leg. Two juveniles had a blastema at the distal end of coxa 1 of their 4th left Walking leg. One juvenile had a blastema at the distal end of coxa 1 of the 1stleft Walking leg (See Table 3).

3.4 *Pentapycnon charcoti*

Six juveniles and four adults were examined. None of these animals showed any signs of regeneration of limbs. These ten specimens were the total of all members of this species in the NMNH collections.

Table 2 Summarizing the results for *N. charcoti*. The 9 missing walking legs all appeared to have been autotomized at the junction of coxa 1 and coxa 2.

Left legs	Number of limbs with a blastema	Right Legs	Number of limbs with a blastema
(I)	1	(I)	0
(II)	1	(II)	1
(III)	2	(III)	2
(IV)	1	(IV)	2

Table 3 Summarizing the results for *Colossendeis tortipalpus.* The 5 missing walking legs all appeared to have been autotomized at the junction of coxa 1 and coxa 2.

Left legs	Number of limbs with a blastema	Right Legs	Number of limbs with a blastema
(I)	1	(I)	1
(II)	0	(II)	0
(III)	0	(III)	0
(IV)	2	(IV)	1

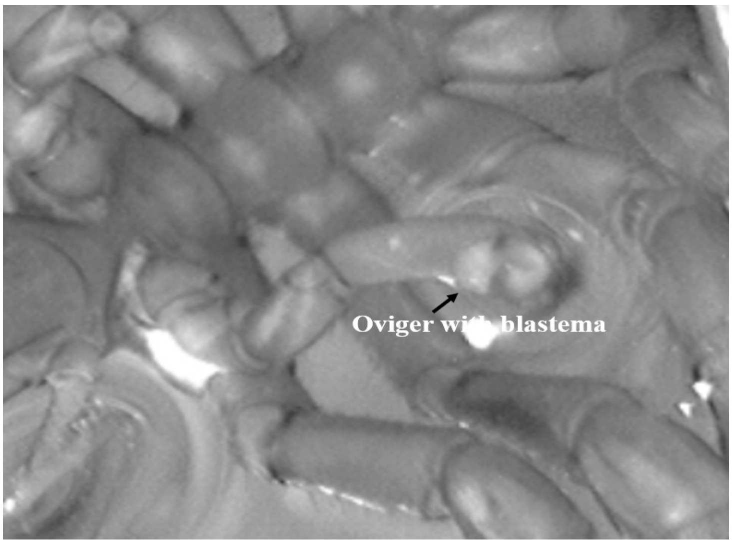

Fig. 3 The left ovigerous appendage of *Colossendeis tortipalpus* with the blastema at the distal end of the third segment of the right ovigerous appendage.

4 Discussion

In his book, Pycnogonids, P. E. King (1973) states “If ambulatory limbs are broken off, the rupture usually occurs between the second and third segments of the limb in *Nymphon gracile*. Pycnogonids haveconsiderable powers of regeneration in the juvenile stages and when a break occurs the regenerative process is very rapid. After the stub of the ruptured limb is sealed off the new rudimentary limb is formed within the cuticle of the remaining part of the limb and is completed at a molt”. In this study the PBP in *N. australe, N. charcoti,* and *C. tortipalpus* is between the first and second coxa segments. The difference may simply be a species specific variation. In crustaceans, this autotomy plane is characterized by the presence of a connective tissue sheath and autotomy diaphragm the function of which is to minimize the loss of body fluids when a limb is autotomized (Skinner and Cook, 1991).

The observed growth of the blastema, starting within the distal most remaining limb segment and then extending beyond this within a protective cuticle is observed in the Chelicerata, *Limulus,* and the *Crustacea* (Gross, 1969; Maruzzo, et al., 2005; Maruzzo and Bortolin, 2013). The presence of regenerating limbs with all segments present but smaller than the corresponding limbs on the same animal as seen in this study, indicates that regeneration requires more than one molt cycle to produce a fully formed and normal size regenerated walking limb (Gross, 1969; Maruzzo, et al., 2005; Maruzzo and Bortolin, 2013).

The total number of specimens of *Pentapycnon charcoti* was relatively small, 10 animals. There was an average of 40% of the specimens from the other three species displaying some degree of regeneration. We could therefore expect about four animals showing some degree of regeneration. The absence of any indication of regeneration including blastema formation may be simply due to the small sample size. Alternately it may be that this genus lacks the ability to autotomize and regenerate limbs. Consequently those individuals losing a limb or part of a limb simply do not survive the trauma.

Adult animals which have reached their final size and have stopped molting do not show evidence of regenerative ability according to Fage (1949) and Helfer and Schlottke (1935). In this study evidence of regeneration in adult *N. austral*e in the form of blastema formation was observed.

Our results agree with Fleming et al. (2007) in that only limb regeneration occurs in the arthropods. The ability to autotomize damaged limbs and regenerate a replacement has obvious selective advantages for any animal (Maginnis, 2006).

Regeneration has been in the ovigerous appendages of *C. tortipalpus* and the cheliphores, *N. australe* respectively. Chelophore regeneration had been inferred previously by Child (1979).

Acknowledgements

Austin Patrick Harlow is acknowledged for assistance with the photomicrographs.

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