



Article

Description of Limb Anomalies Resulting from Molt Irregularities in *Ammothea hilgendorfi* (Pycnogonida: Ammotheidae)

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Abstract: Limb anomalies are widespread and diversified in arthropods. From trilobites to insects, they range from the loss to the addition or fusion of legs and may appear congenitally or be induced experimentally (e.g., amputation or injury). Basal chelicerates pycnogonids, or sea spiders, also show deformities. Despite being understudied compared to other arthropods, quite a high diversity of limb malformations has been reported in the literature. The present study reports the leg anomalies of two adult females *Ammothea hilgendorfi* (Böhm, 1879) observed with duplicated leg podomeres. Both individuals were described ethologically and morphologically. Although the current knowledge on pycnogonids is limited, the anomaly is likely due to a problem in the molting process; the specimens were unable to totally remove their old exuviae, which then stacked after the proximal leg segments. The second specimen also showed other leg deformities, hinting at a problem during the molting process itself. The discussion emphasizes that understanding how pycnogonids normally molt would not only help our understanding of how the abnormal patterns appeared but also put pycnogonids into perspective with other arthropods, a phylum in which they have a key taxonomic position.

Keywords: pantopoda; sea spider; pycnogonid; morphological variation; malformation; molting



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1. Introduction

Pycnogonids, also known as pantopods or sea spiders, are an understudied class of marine chelicerates containing approximately 1400 described species [1]. They are characterized by a slender body, a large proboscis, a tiny abdomen, and a pair of ovigers [2]. Most species have eight locomotor appendages (i.e., legs), but some fossils or extant species naturally have ten or twelve [3,4]. For each species, the leg is normally composed of eight segments (Figure 1): three coxae, a femur, two tibiae, a tarsus and a propodus (see [5] for comparison and homology with other chelicerate groups). The terminal segment is equipped with a terminal claw and, when present, two auxiliary claws used to grasp onto surfaces [6].

Due to the reduction of the pycnogonid body, many of its internal organs have extended into the limbs [2]. Therefore, its legs serve not only a locomotor function, but also physiological functions, as the digestive tract extends into the proximal leg podomeres [7]. The same is true for both male and female reproductive tracts, which open in a gonopore located ventrally on the second coxa [8,9] on all pairs of legs except in some genera [10]. Gravid females store their eggs in femurs that become thicker. Mature males, on the other hand, use their cement glands located on the femur (plus the first tibia in a few species) to produce a glue that groups the eggs together [10]. The exact position, number, and structure of cement glands are subjected to high variability according to the phylogenetic position of the species [11]. After reproduction, the male carries the eggs on its ovigers (i.e., exclusive paternal care) [12].

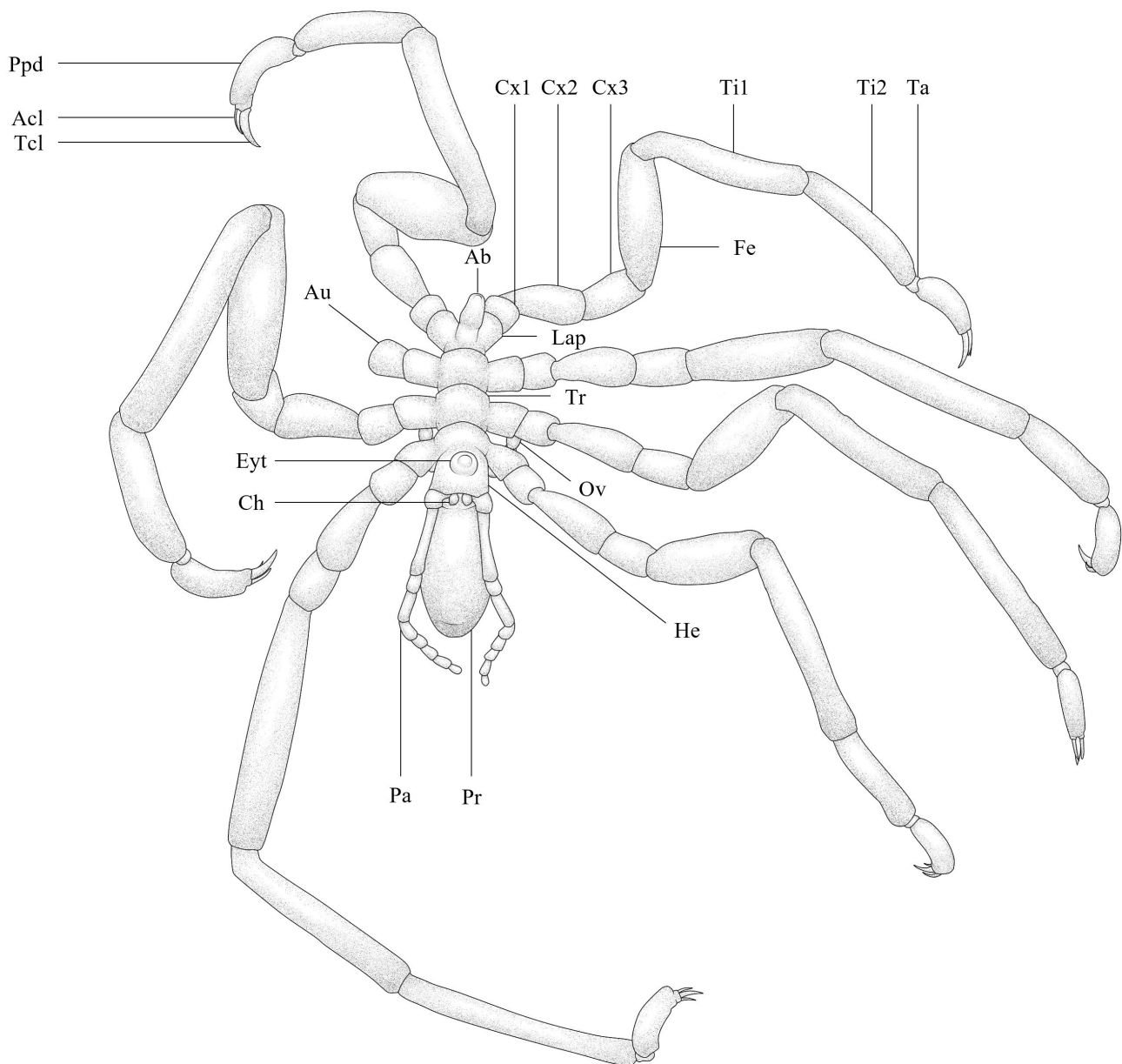


Figure 1. Normal adult female of *Ammothea hilgendorfi*. Ab: abdomen; Acl: auxiliary claw; Au: auto-tomized leg (between coxa 1 and coxa 2); Ch: chelifore (atrophied in that species); Cx1: coxa 1; Cx2: coxa 2; Cx3: coxa 3; Eyt: eye tubercle; Fe: femur; He: head; Lap: lateral process; Ov: oviger (inserted ventrally); Pa: palp; Pr: proboscis; Ppd: propodus; Ta: tarsus; Tcl: terminal claw; Ti1: tibia 1; Ti2: tibia 2; Tr: trunk.

Like all arthropods, pycnogonids have a cuticle made of chitin and undergo molting as they grow [13]. Most described sea spiders are hemianamorphic, meaning that the first molts are anamorphic (i.e., resulting in segment addition during the larval stage) (e.g., [14]), and then they become epimorphic (i.e., no segment addition during the juvenile stage) [15]. During the epimorphic molts of *Nymphon gracile* Leach, 1814 [16,17], and *Pycnogonum litorale* (Strøm, 1762) [18,19], the old cuticle breaks off in several pieces. Every leg exuvia is shed like a sleeve and the body cuticle splits into a ventral and a dorsal part. It seems that it was already the case in the fossil species *Palaeomarachne granulata* Rudkin, Cuggy, Young & Thompson † [20]. Some adult *P. litorale* may grow without even molting [19], as they periodically “desquamate” portions of damaged cuticle [20].

Malformations are not uncommon in arthropods. They have been documented in all taxa including Crustacea (e.g., [21]), Myriapoda (e.g., [22]), and Chelicerata (e.g., [23]). Pycnogonida is no exception (e.g., [24]).

Arthropods as old as trilobites already showed malformations (e.g., [25,26]). They were either attributed to teratological maldevelopment, pathologies, or post-injury regeneration. Regenerating tissues are indeed mutually independent to some degree, which may lead to mistakes in healing processes [27]. Documentation on extant arthropods anomalies flourishes and covers insects (e.g., [28–30]), myriapods (e.g., [22,31]), and crustaceans (e.g., [28,32,33]). Notably, Hesse-Honegger and Wallimann (2008) collected true bugs (Heteroptera) close to nuclear plants that were missing a tibia and/or femur (among many other types of anomalies) [34].

In Chelicerata, anomalies of locomotor appendages are well documented and have their own terminology. They include oligomely (i.e., reduction in the number of legs; e.g., [35]), polymely (i.e., additional appendage(s)), symely (i.e., fusion of facing legs), heterosymely (i.e., fusion of adjacent legs on the same side of the body; e.g., [36]), schistomely (i.e., bifurcation of legs), or a complex of these abnormalities [37–39]. These malformations can be induced by exposing embryos to increases in temperature [40,41] or alternating high and low temperatures [37,39]. Instances of malformed opisthosoma and epigynum have also been reported in spiders, which may be collected in nature [42,43] or induced by temperature variations [23]. Other anomalies in spiders include gynandromorphy (i.e., having both male and female features) [44–46] and color anomalies [47].

Missing legs can be observed in pycnogonid populations but are often the result of autotomy between the first and second coxae (Figure 1) [48], after which the lost limb can regenerate in a later molt [49]. On the other hand, it is considered an oligomelic malformation when no remnants of the missing leg are observable (e.g., a six-legged *Callipallene brevisrostris* (Johnston, 1837) [50,51] and an asymmetrical seven-legged *P. litorale* [52]). Oligomelic sea spiders can also be experimentally induced by amputating the posterior trunk [53]. Loeb (1905) mentioned a *Phoxichilidium femoratum* (Rathke, 1799) [50] that regenerated its posterior trunk into a limb-like structure [54], and Scholtz and Brenneis (2016) observed a *P. litorale* growing an additional leg after an injury to its trunk [55]. Other anomalies in pycnogonids have been documented, including a postlarval instar *Nymphonella tapetis* Ohshima, 1927 [50], with six legs, including one trifold [56]; a postlarva *P. femoratum* missing a trunk segment, with the previous one only bearing one abnormal leg [57]; a nine-legged *Anoplodactylus petiolatus* (Krøyer, 1844) [50] in which the last three legs were fused; a *Chaetonymphon spinosum* [accepted as *Nymphon hirtum* Fabricius, 1780 [58]] with a bifurcated abdomen; and another *C. spinosum* with an aberrant chelifore [24]. Gynandromorphy has also been described [59,60]. Finally, a specimen of *Ammothea hilgendorfi* (Böhm, 1879) [50] with a bifurcated abdomen was recently collected in France [61].

A. hilgendorfi is a pycnogonid native to the North Pacific Ocean that was recently introduced in Europe (e.g., [62]). This study is part of a bigger one monitoring the Belgian population of that species [63]. It describes two specimens of *A. hilgendorfi* with a unique limb anomaly, externally appearing as a duplication, in tandem, of their leg podomeres (i.e., leg segment tandem duplication (LSTD)). The morphology of Malformed Individuals 1 and 2 (MI1 and MI2) has been characterized using optic and electronic microscopy and its impact on locomotion has been filmed with a camera.

2. Materials and Methods

Both specimens were collected on a rocky wave breaker covered with *Magallana gigas* (Thunberg, 1793) [64] oysters, along with other pycnogonids of the same species, in Knokke, Belgium (51°21'16" N; 3°16'54" E) [63]. MI1 was collected on 3 April 2022, and was identified as a juvenile *A. hilgendorfi* based on the oviger development in [65]. It was kept alive in the aquariums of the University of Mons (UMONS, Belgium) at a constant 15 °C, 35 PSU, under a 12:12 day–night cycle. It first looked normal and behaved like the other specimens simultaneously collected. However, after 15 days, it showed unusual behavior,

and was identified as an adult female based on [65]. MI2 was collected on 30 November 2023, and was identified as an adult female *A. hilgendorfi*.

The specimens were observed for 24 h inside 10 × 7 × 6 cm rectangular mesocosms with a suitable substratum for the animals to grasp onto (i.e., *M. gigas* shell pieces on a mosquito net), then filmed inside an empty plastic jar with an Olympus Tough TG-6 camera (Video S1). MI1 was fixated in Bouin's solution (75% picric acid, 25% formaldehyde), replaced with 70% ethanol 24 h later. Then, it was dehydrated using increasing ethanol bath concentrations and critical point drying. The sample was rinsed twice with 90% ethanol for 30 min and placed in a 100% pure ethanol bath for 1 h. This was then replaced by liquid CO₂ inside a SPI-Dry Critical Point Dryer pressure bomb (six 20 min baths) and dried by exceeding the critical point of CO₂ (31 °C and 74 bar). The sample was then covered with a 5 Å layer of 40% gold and 60% palladium using a JEOL JFC-1100E pre-vacuum enclosure. It was finally observed with Scanning Electron Microscopy (SEM) (Jeol, JSM-7200F). A normal adult female *A. hilgendorfi* underwent the same protocol for comparison. MI2 was fixed in 70% ethanol. Afterwards, several photographs were taken under a Keyence VHX-970F digital microscope, focusing first on each abnormal leg segment individually, stitching them together with Clip Studio ver. 1.13.2, and then on each observable irregularity.

MI1 underwent precise biometric analyses: the SEM camera was tilted to be orthogonal to each individual segment before a photo was taken. Then, their length at the center and width at the largest point were measured using ImageJ (Java 1.8.0_312). The segments were then identified based on biometrics and their positions on legs. The length and width of the proximal and distal copies of each duplicated segment were compared to each other using a Wilcoxon matched-pair signed-rank test (alpha = 5%) using Prism 5.0.0.

3. Results

3.1. Malformed Individual 1 (MI1)

The malformation externally appeared as an extra set of segments in six of the eight legs (Figure 2), showing between 1 and 4 additional segments per leg (Table 1). An example of an abnormal leg can be seen in Figure 3. While no significance was found between the length of both segment copies ($p = 0.49$), the width of the distal copies was significantly smaller than the proximal ones ($p = 0.0084$).

Table 1. Number of segments counted on each leg of MI1 and their names, based on their positions and similarities to eight-segmented legs. Each last coxa 1 per leg is highlighted in bold. The cell background of normal legs is highlighted in light grey.

	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
1st left	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus				
2nd left	Coxa 1	Coxa 2	Coxa 3	Femur	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 1	Tarsus	Propodus
3rd left	Coxa 1	Coxa 2	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus		
4th left	Coxa 1	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus			
1st right	Coxa 1	Coxa 2	Coxa 3	Femur	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus
2nd right	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus				
3rd right	Coxa 1	Coxa 2	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus		
4th right	Coxa 1	Coxa 2	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus		

The additional segments were observed to be duplicated as groups, rather than directly following their counterparts, as, for instance, in the third left leg where the order was [Cx1-Cx2]-[Cx1-Cx2] instead of [Cx1]-[Cx1]-[Cx2]-[Cx2] (i.e., LSTD). This was verified by coxae 2 being longer than coxae 1 in the normal and abnormal legs (Table 2). SEM photographs revealed cuticle tearing at the base of all distal copies of coxae 1 (Figure 4), reminding us of the proximal part of the leg exuviae being shed like a sleeve in *P. litorale* and *N. gracile* [17,19]. Gonopores, typically found ventrally on each of the second coxae in *A. hilgendorfi* [11], were, however, absent in the distal copies.

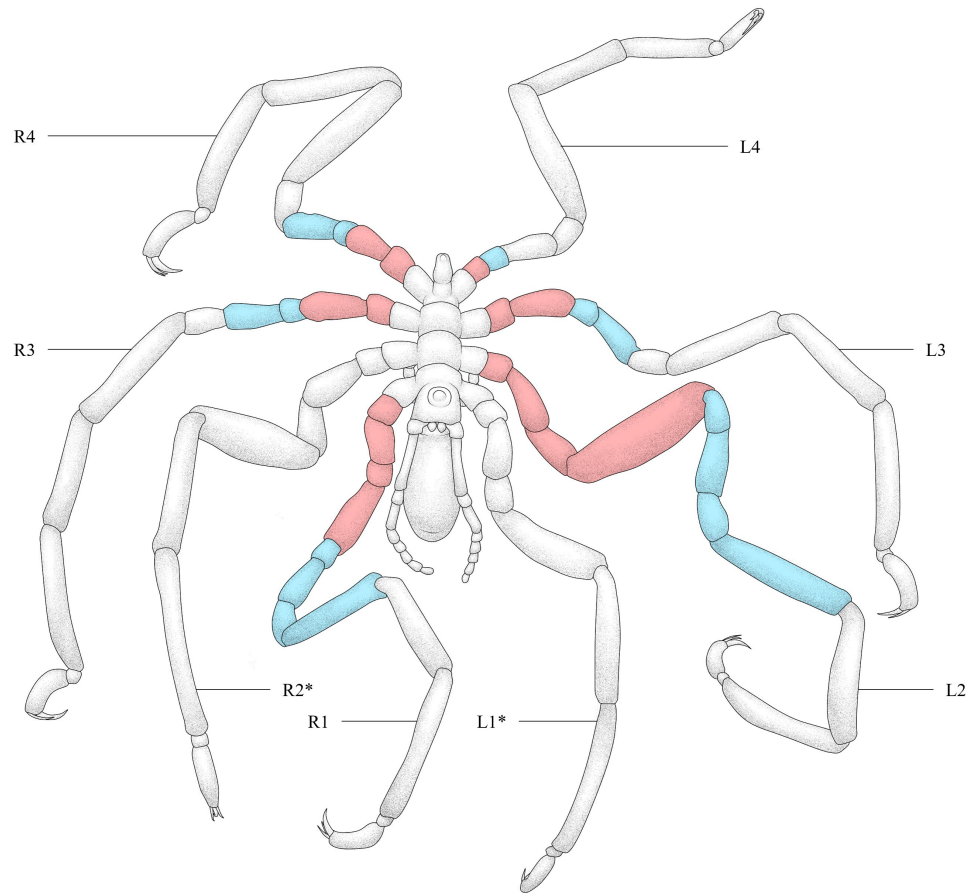


Figure 2. Abnormal adult female of *Ammothera hilgendorfi* (MI1). L1: first left leg; L2: second left leg; L3: third left leg; L4: fourth left leg; R1: first right leg; R2: second right leg; R3: third right leg; R4: fourth right leg. Red: proximal copies of duplicated segments; blue: distal copies. The * indicate the two normal legs.

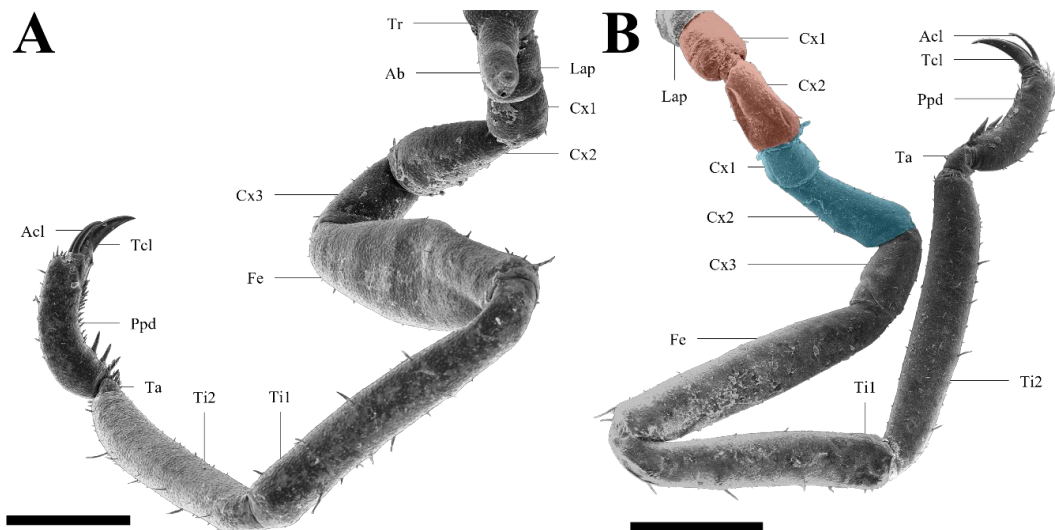


Figure 3. Fourth right leg of adult female *A. hilgendorfi* under Scanning Electron Microscopy (SEM). (A) normal leg; (B) example of malformed leg. Ab: abdomen; Acl: auxiliary claw; Cx1: coxa 1; Cx2: coxa 2; Cx3: coxa 3; Fe: femur; Lap: lateral process; Ppd: propodus; Ta: tarsus; Tcl: terminal claw; Ti1: tibia 1; Ti2: tibia 2; Tr: trunk. Red: proximal copies of the duplicated segments; blue: distal copies. Scale bars: 1 mm.

Table 2. Mean \pm standard deviation of the length at the center, and the width at the largest point, of each segment of each leg in MI1 (in μm).

	Coxa 1	Coxa 2	Coxa 3	Femur	Tibia 1	Tibia 2	Tarsus	Propodus
N	14	13	10	10	8	8	8	8
Length	381 \pm 83	845 \pm 131	545 \pm 77	1982 \pm 439	2013 \pm 372	2382 \pm 540	197 \pm 31	838 \pm 77
Width	381 \pm 34	417 \pm 39	395 \pm 29	488 \pm 113	390 \pm 25	358 \pm 55	208 \pm 16	287 \pm 36

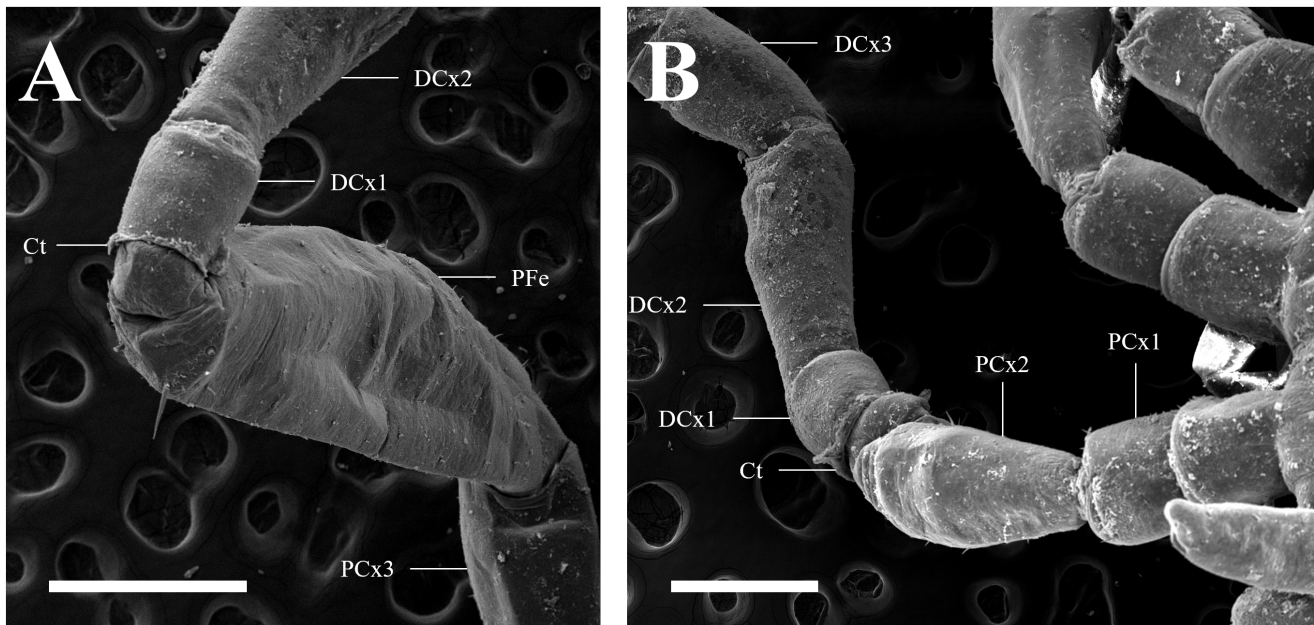


Figure 4. Second left leg (A) and third left leg (B) of MI1 observed in SEM. Ct: cuticle tearing; DCx1: distal coxa 1; DCx2: distal coxa 2; DCx3: distal coxa 3; PCx1: proximal coxa 1; PCx2: proximal coxa 2; PCx3: proximal coxa 3; PFe: proximal femur. Scale bars: 500 μm .

It can be seen on Video S1 that the distal ends of abnormal legs look white and empty. The video also shows that, despite being able to move its limbs independently, MI1 seemed unable to coordinate its movements. The terminal claws were disorientated: instead of grasping substrates below the body, these grasped substrates positioned above the animal.

3.2. Malformed Individual 2 (MI2)

MI2 showed only one aberrant limb (Figures 5 and 6A), yet the anomaly was much deeper. At least all three coxae externally appeared as duplicated in tandem once, with the presence of two gonopores confirming the position of both coxae 2 (Figure 6B,C). However, four additional anomalies were noted on that single leg. First, a putative third coxa grew after yet another coxa 3 (see “Cx3?” in Figure 5). Its dimensions disprove the possibility of a coxa 1, which are much smaller, and it did not have a gonopore, unlike the other coxae 2 of that leg. Second, right after the proximal coxae was noted an unusual segment (see “Fe?” in Figure 5). Although its position hinted at a femur, it did not look like a femur or any normal segments whatsoever. It was, indeed, more opaque, the digestive tract being barely visible through it. In fact, its constricted appearance was similar to what was noted in [48] (see their Figures 4 and 5), which is an elongated regenerate with joints between future segments (Figure 6E). Third, the yellowish cuticle pigmentation suddenly stopped at the beginning of tibia 1, with a stump-shaped structure inside the leg itself (see “Pge” in Figure 5). Beyond that, tibia 1 contained structures that looked like the regenerating legs observed in [48] (see their Figure 11). The following segments seemed empty, as if they only consisted of external cuticle. Fourth, a terminal and an auxiliary claw (Figure 6D)

were visible between both tibias, inside them. The claws at the distal end of the leg, on the other hand, did not seem functional as they were almost falling off the propodus (Figure 5).

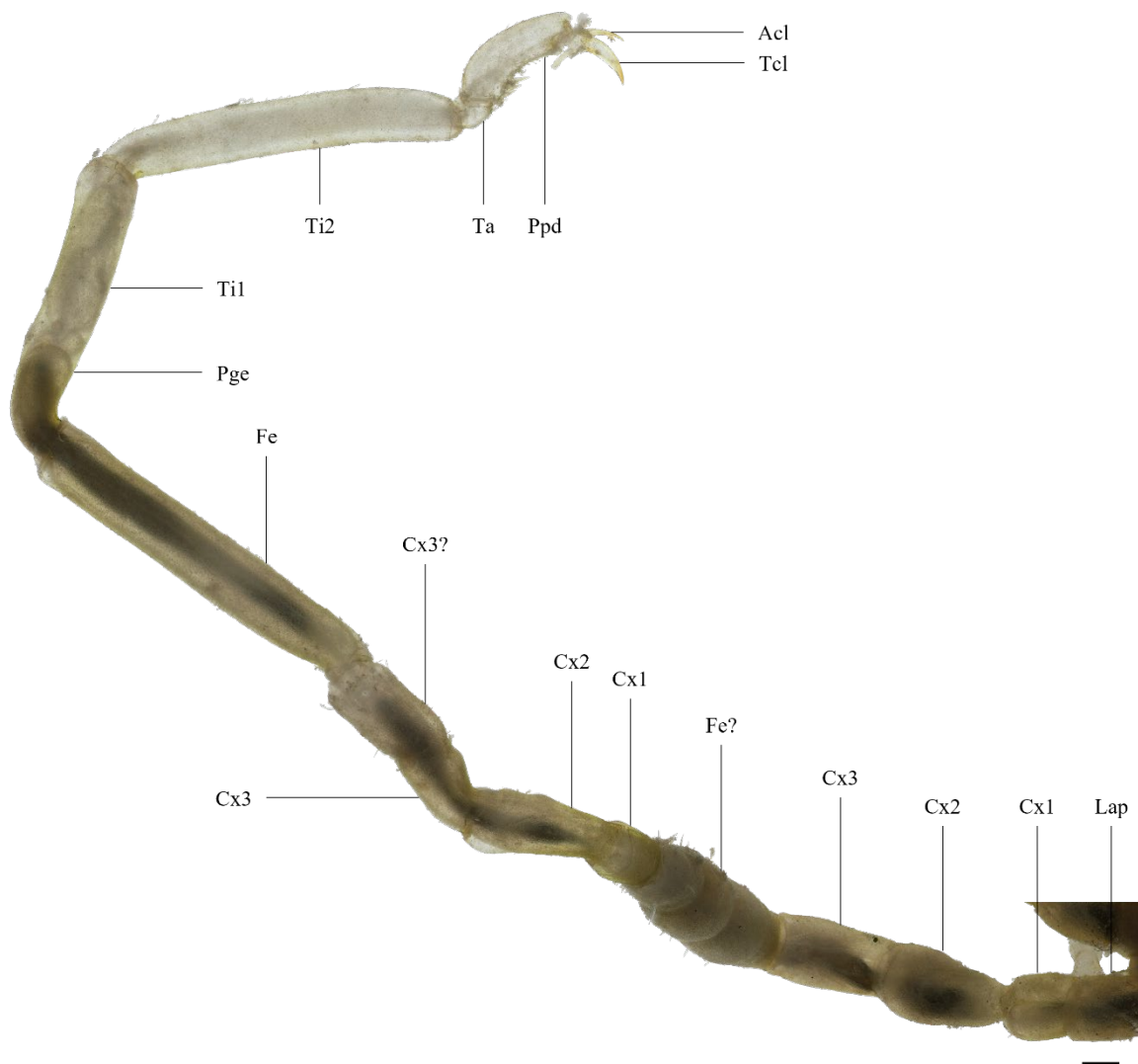


Figure 5. Dorsal view of the abnormal leg (second left) of MI2 photographed with a Keyence VHX-970F digital microscope. Acl: auxiliary claw; Cx1: coxa 1; Cx2: coxa 2; Cx3: coxa 3; Cx3?: putative coxa 3; Fe: femur; Fe?: putative femur; Lap: lateral process; Pge: pigmentation end; Ppd: propodus; Ta: tarsus; Tcl: terminal claw; Ti1: tibia 1; Ti2: tibia 2. Magnification: X200. Scale bar: 0.25 mm.

Video S1 shows that, unlike MI1, MI2 behaved normally, using all its legs to walk except for the abnormal one. It looked unable to move the abnormal limb actively, which remained immobile above the others. Still, that did not incapacitate the whole animal's movements, and it could walk just as if the malformed leg was missing.

In [63], only two malformed *A. hilgendorfi* were detected among 1144 individuals, suggesting an anomaly prevalence rate of ~0.2% in the Belgian population of that species.

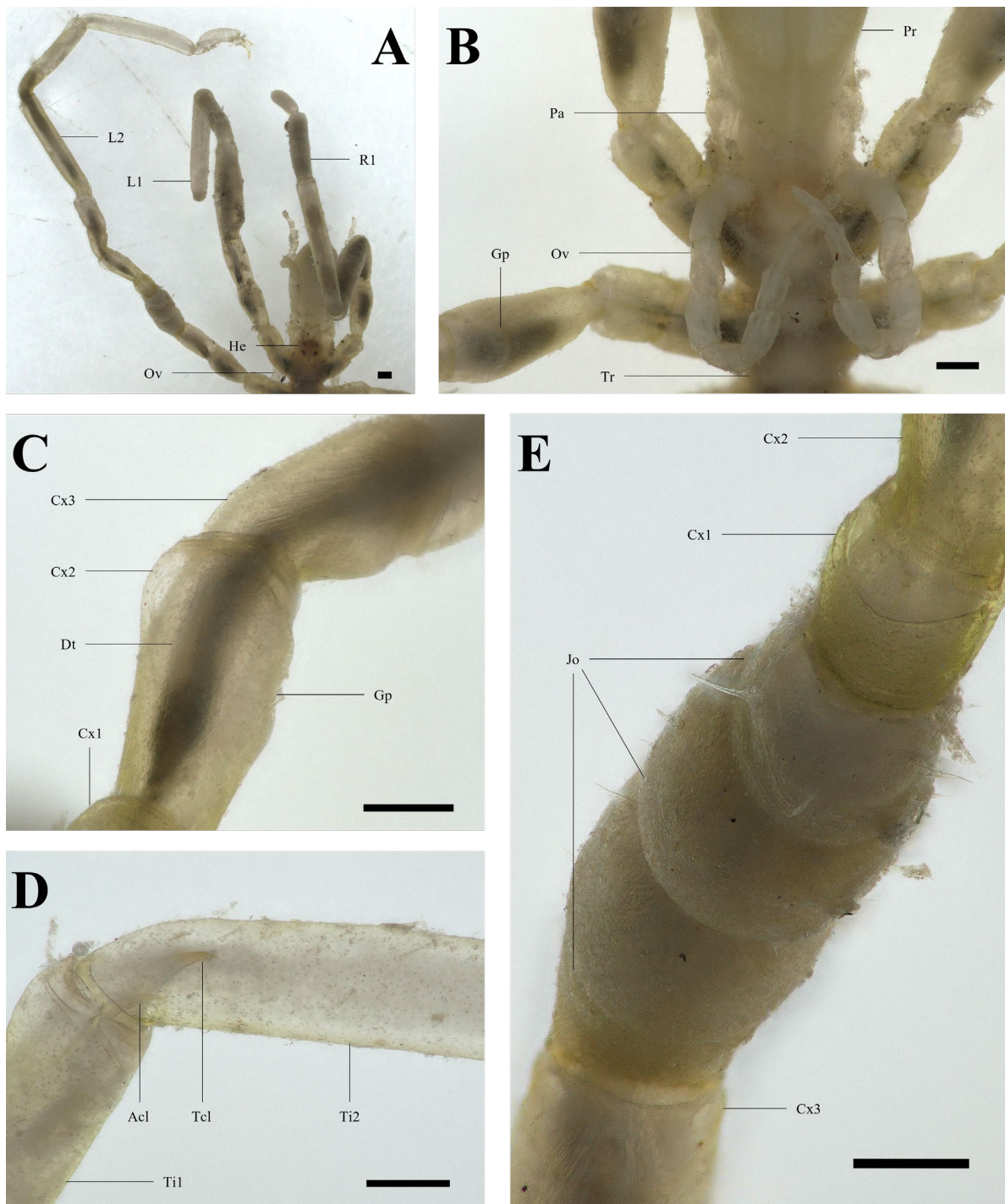


Figure 6. Features of the abnormal leg of MI2, photographed with a Keyence VHX-970F digital microscope. **(A)** Anterior dorsal view of the specimen. He: head; L1: first left leg (autotomized after tarsus); L2: second left leg (malformed), Ov: oviger; R1: first right leg. **(B)** Ventral view of the specimen and proximal leg segments. Gp: gonopore (ventrally on the second coxa); Ov: oviger; Pa: palp; Pr: proboscis; Tr: trunk. **(C)** Lateral view of the distal copy of the second coxa. Cx1: distal coxa 1; Cx2: distal coxa 2; Cx3: distal coxa 3; Dt: digestive tract; Gp: gonopore. **(D)** Ventral view of claws inside empty distal segments. Acl: auxiliary claw; Tcl: terminal claw; Ti1: tibia 1; Ti2: tibia 2. **(E)** Dorsal view of the putative femur. Cx1: distal copy of coxa 1; Cx2: distal copy of coxa 2; Cx3: proximal copy of coxa 3; Jo: putative joints of future segments appearing as constrictions. Magnifications: X30 in **(A)**; X100 in **(B)**; X200 in **(C–E)**. Scale bars: 0.25 mm.

4. Discussion

An anomaly identical to the observed LSTD, or the four others described in MI2, could not be found in the literature regardless of the taxon considered, whether it be pycnogonids, chelicerates, or the entire arthropod phylum. In fact, many anomalies described in the literature concern entire legs and are rarely limited to a few segments (e.g., Heteroptera missing a femur and/or tibia [34]).

However, this phenomenon reminds us of the molting process of other sea spiders, in which the leg cuticle is cast like a sleeve [17,19]. The cuticle tearing noticed in Figure 4 would then be the proximal end of the exuviae being removed, while the transparent distal end in Video S1 would be due to them being moved beyond the internal organs. This is emphasized by MI1 being identified first as a normal juvenile and then as an abnormal adult, suggesting that the anomaly arises from a molt. The proximal copies of the duplicated podomeres in MI1 were found to be significantly wider, which makes sense considering that they are the newly formed podomeres and that pycnogonids become bigger after molting [19]. Plus, it was identified in several sea spider species that gonopores appear at the adult stage [66]. From the examination of several last-stage juveniles, this seems to be the case in *A. hilgendorfi* as well. This is consistent with the fact that the distal copies of coxae 2 did not feature gonopores, because they would have been contemporary to the last pre-adult stage.

Although the life history of MI2 was impossible to trace, the empty distal end of its malformed leg points to the same cause. The claws visible through the cuticle (Figure 6D) would be the newly formed ones, while the claws almost falling off at the top would be old cuticle, removed during a normal molt.

Even if the anomaly likely results from a molt, it is improbable that the observed phenomenon is usual and expected in every *A. hilgendorfi*. Unfortunately, the molting process of sea spiders has been poorly studied in the literature, making comparisons with normal individuals difficult. However, the specimens were observed for 24 h, and neither their anomaly nor ethology changed, despite their access to a proper substrate to lock their leg exuviae on. Since many empty leg cuticles were found at the bottom of aquariums in which other *A. hilgendorfi* not showing any anomalies were maintained, this species likely molts quickly. As the ovigerous legs of MI1 had already molted, and its two legs looked normal, the observed LSTD probably arose from the inability of the specimen to completely cast its old cuticle during the molting process. In fact, the state in which MI1 was found might resemble what happens during an expected regular pycnogonid molt. Therefore, while the malformation cannot be considered a “podomere duplication” sensu stricto, as it is only exuviae, it behaves effectively as if this was the case. Indeed, although the locomotion of MI1’s legs was not altered independently, the locomotion of the whole animal was compromised. Such a phenomenon occurring in the wild would likely result in a fitness close to zero (i.e., low survival). Regarding MI2, its only malformed leg did not seem to impact it, similarly to how autotomized specimens are viable in the wild [49].

LSTD was not the only anomaly in MI2. The abnormal putative femur reminds us of a regenerating and constricted segment [48], hinting at the incapacity of this leg to molt completely in the first place, that maybe even occurred during an anterior molt. The putative coxa 3, right after another coxa 3, might as well translate to an incompletely removed cuticle sleeve in a molt previous to the last one. Petrova and Bogomolova (2023) observed that regenerating legs fold inside amputation stumps [48], which might match the structures inside the first tibia of MI2, although external morphology analyses alone cannot assess them with certitude. The doubled gonopores also raise questions about both the anomaly origin and the ontogenetic development of *A. hilgendorfi*. As mentioned above, *A. hilgendorfi* seem to grow a gonopore during their juvenile-to-adult molt. While adult *P. litorale* stop molting (i.e., terminal anecydysis) [67], either adult *A. hilgendorfi* do continue molting or the anomaly arises from the last molt mistakenly triggering twice in that leg. Further research on their life cycle is necessary to address these hypotheses.

It must be mentioned that the context in which this study took place was not in trying to spot malformed individuals. Both cases here described were extreme and noticed “by chance”. Thus, there may lie in the Belgian population of *A. hilgendorfi* more subtle leg irregularities which would have been overlooked here. The counted ~0.2% prevalence of abnormal individuals should then be considered a minimum.

That prevalence, although likely underestimated, remains in the range of malformation ratios noted for different arthropod groups. For example, Hesse-Honegger and Wallimann (2008) collected 16,000+ Heteroptera in several locations and noted morphological anomalies at a ratio down to 1–3% in undisturbed habitats, and up to 22% close to a nuclear reprocessing plant [34]. In crustaceans, Rady (2022) found in the Suez Canal 28/269 (~10.4%) *Portunus segnis* (Forskål, 1775) [68] crabs with a malformation in any body part [21]. In chelicerates, Palmgren (1979) counted a ratio of 1/17,000 (~0.006%) gynandromorph spiders (several species included) [45]. In pycnogonids, Bouvier (1914) investigated 3268 *P. litorale*, but found only one abnormal specimen (~0.03%) [52]. On the other hand, Lotz and Bückmann (1968) observed 22/67 *P. litorale* correctly molting at least once in a period of ten months (~33%) [19]. That ratio is much greater than the 0.2% of the present study, which is another hint at the fact that MI1 and MI2 were abnormal.

The alien trait of *A. hilgendorfi* should not be neglected. Indeed, the introduction of a new species generally results in genetic bottlenecks in the colonized area, resulting in more homozygotes in the alien population [69]. This is associated with their lower fitness [70], and homozygosity has been linked many times to pathologic phenotypes (e.g., [71–73]). If that is so, the prevalence of leg anomalies—or any other malformations—would likely be higher in non-native populations of *A. hilgendorfi*. A study specifically looking for all malformations in several *A. hilgendorfi* populations (i.e., native vs. non-native) would be of interest to assess the consequences of its introduction for the species itself.

Finally, nothing is known about their molt dynamics (i.e., time required, different steps, etc.). Understanding how pycnogonids molt would not only help our understanding of how the abnormal patterns appeared but also put pycnogonids into perspective with other arthropods, a phylum in which they show a key taxonomic position.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/arthropoda2020012/s1>, Video S1: Behavior of the malformed specimens.

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Data Availability Statement: The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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