

Healing and regeneration in horseshoe crab, with possible application to trilobite injuries

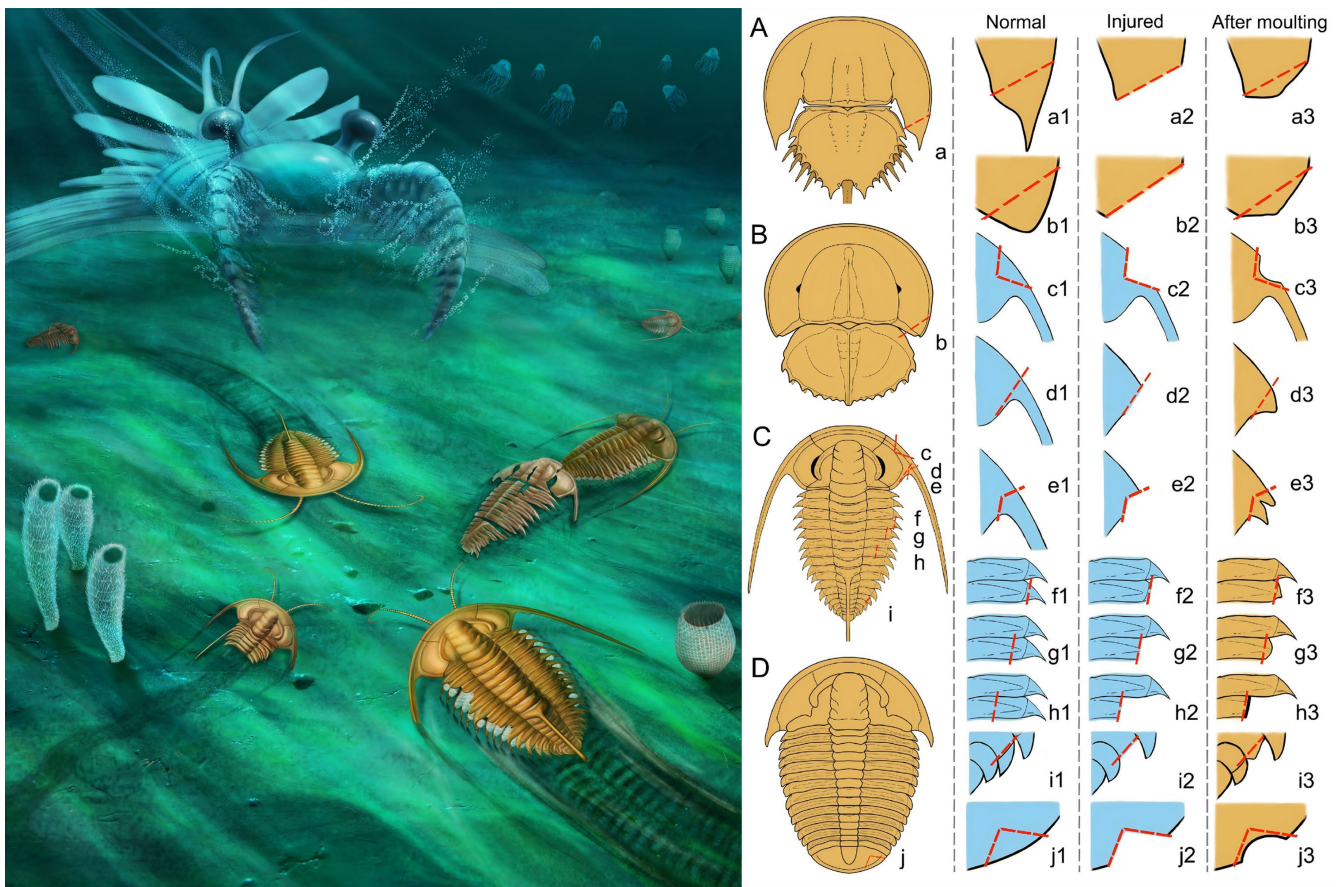
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Graphical Abstract



Highlights:

- The experimental results utilizing the extant horseshoe crab demonstrated that the newly-formed exoskeleton only regenerates and modifies the shape of the wound after moulting.
- The results showed that trilobites that had not moulted after injury were rarely identified, as the sharp wounds were indistinguishable from post-mortem breakage.
- It suggests that the majority of the trilobite injuries in the fossil record may have been missed, including successful predation events, lethal injuries, and damage incurred during the adult stage.
- This recognition biases leading to the underestimation of trilobite injury frequency.

Healing and regeneration in horseshoe crab, with possible application to trilobite injuries

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Abstract: Trilobites exhibiting injuries are ideal palaeontological archives for Palaeozoic predator-prey interactions. However, the current methods for diagnosing trilobite injuries primarily depend on the various morphological features at the edges of the injured exoskeleton, without evaluating the process and timing of morphological changes. Here we performed an amputation experiment utilizing the extant horseshoe crab as a proxy of trilobites to study the morphological changes after injury. The results show that: the wound closure was accomplished by forming hemolymph clotting on the wound surface in a short time. And then, a thin cuticle layer was growing on the inner side of wounded exoskeleton, with the retention of a sharp outline of the wound. Particularly, the newly-formed exoskeleton only regenerates and modifies the shape of the wound subsequent to moulting. After rechecking 409 abnormal trilobites documented in the published literature, we recognized that the injuries with sharp exoskeleton edge are very rare in the fossil record, and 95% of injured trilobites with clearly identifiable scars had undergone at least one subsequent ecdysis. This indicates that the majority of the injuries were not identified, as the sharp wounds were indistinguishable from post-mortem breakage. These unidentified injuries may encompass successful predation events, lethal injuries, and damage incurred during the adult stage, none of which exhibit signs of moulting. Although we are currently unable to deal with this recognition bias, through injury experiments on living arthropods, our understanding of the damage types and repair processes of trilobites will be further enriched.

Keywords: trilobite; predation; injury; horseshoe crab; experimental palaeontology

1. Introduction

Predation played an important role in the evolutionary 'arms race' (Dawkins and Krebs 1979; Mcmenamin 1987; Bengtson 2002) that contributed to a dramatic increase in diversity and abundance of biomineralized organisms during the Ediacaran radiation and Cambrian Explosion (Bengtson and Yue 1992; Smith and Harper 2013; Zhang *et al.* 2014; Wood *et al.* 2019). Trilobites, one of the most iconic arthropods of those arthropods that have a hard exoskeletons, were a direct product of this evolutionary 'arms race', well documented by a variety of durophagous injuries on hard exoskeletons (Fatka *et al.* 2021; Bicknell *et al.* 2022a, 2022b, 2022c; Zong and Bicknell 2022). Although they were a prominent component of Paleozoic marine ecosystems (Hughes 2008; Kraft 2023), the frequency of injuries on trilobites within most populations is remarkably low (Morris and Jenkins 1985). Snajdr observed abnormalities in only about 0.5% of encrinurine trilobites (Snajdr 1985). Babcock reported frequencies

ranging from 2% to more than 5% in his trilobite collections (Babcock 2007). More recent studies have calculated higher repair frequencies (18%) in trilobites from field collections (Pates and Bicknell 2019) and showed variations of repair frequencies (from 0 to 14%) across different species (Pates *et al.* 2017). The incongruity between the intense predator-prey 'arms race' and the infrequent observation of injured trilobites may be attributable to a combination of factors, such as taphonomic bias (Babcock 2007; Bicknell and Paterson 2017), the paleoclimatic and the paleogeographic context (Vinn 2017), the artifacts of sampling (Vinn 2017), the examination of separated sclerites compared to articulated exoskeletons, moult ensembles compared to carcasses (Babcock 2003), differing susceptibilities to malformations among various groups (Babcock 2007), and potential ambiguous recognition of injuries on trilobites. Moreover, the low frequency of injuries might mainly result from successfully executed predation (Morris and Jenkins 1985; Capasso and Caramiello 1996; Bicknell and Paterson 2017; Pates and Bicknell 2019), evidenced by coprolites

(Nedin 1999; Babcock 2003) and gut contents (Zhu *et al.* 2004; Zacaï *et al.* 2016) with abundant trilobites (Nedin 1999; Babcock 2003).

The current diagnoses of trilobite injuries mainly relies on variable morphological characters of the trilobite exoskeleton edges such as the shape of the outline described as arcuate (Leighton 2011), V-shaped (Zamora *et al.* 2011), U-shaped (Bicknell *et al.* 2018), embayed (Jell 1989), or rounded (Morris and Jenkins 1985; Jell 1989), or the variations seen in wound edges often described as thickened (Pratt 1998), raised (Pates and Bicknell 2019), swollen (Jago and Haines 2002), or wrinkled (Morris and Jenkins 1985), or bearing other scar-like structures. However, these diagnoses lack an assessment of the morphological change (the process and occurrence time of morphologic change during injury repair).

A key debate in the literature concerns the temporal occurrence of trilobite exoskeleton alterations in the mentioned diagnoses above. Concerning the frequently recorded diagnoses of trilobite exoskeleton thickening (callus), Sinclair (1947) and Ludvigsen (1977) suggested that the thickening exclusively occurs during subsequent moult stages. In contrast, Owen (1985) proposed that thickening happens before moulting, precisely during the same intermoult period when the injury occurred. To resolve this debate, it is crucial to comprehend the morphological change of the complete repair process in trilobites, as proposed by Ludvigsen (1977), Rudkin (1979), and Morris and Jenkins (1985). Comparison with living animals is necessary to understand the healing process in trilobites (Morris and Jenkins 1985; Hessin 1988; Babcock 1993).

Trilobites are assumed to share affinities with either the chelicerates or mandibulates (Størmer 1941; Wills *et al.* 1994; Budd 2002; Cotton and Braddy 2004; Babcock *et al.* 2017). Recent finding demonstrate that the trilobite hypostome shows a mixed structure such as the xiphosuran epistome accommodates the chelicerae (Albani *et al.* 2024), indicating a potentially stronger phylogenetic affinity with Xiphosura. Recently, it was directly confirmed that chitin was present in the cuticle of trilobites (Bailey *et al.*

2025). Therefore, the morphological changes in trilobite exoskeletons regarding healing and regeneration may be comparable to those of horseshoe crabs. Consequently, utilizing horseshoe crabs as model organisms to investigate the healing processes of trilobites represents an appropriate approach (Speyer 1985; Karim and Westrop 2002; Paterson *et al.* 2007). Here we adopt an amputation experiments that utilize both first instar larvae and late-stage juveniles of horseshoe crabs as a proxy of trilobites.

2. Methods

2.1. Animal-collection and maintenance

Horseshoe crabs living in China are second-grade animals under state protection. The research protocol related to amputation experiment using living juveniles and larvae of the horseshoe crab *Tachypileus tridentatus* (*T. tridentatus*) (Figure 1) was approved by the Committee for the Care and Use of Laboratory Animal Ethics of Northwest University, China (License number, NWU-AWC-20190104H) on January 3rd, 2019. All horseshoe crabs used here were supplied by the Guangxi Institute of Oceanology (GIO) in accordance with a license for artificial propagations and scientific research in *T. tridentatus*. The license was approved by the Department of Agriculture and Rural Affairs of Guangxi Zhuang Autonomous (License number, Guiyu 201800695; term of validity, April 17th, 2018–December 4th, 2021). The experimental horseshoe crabs were maintained at a temperature of 18 °C–28 °C and a salinity of 28‰–31‰. The fresh seawater was replaced every two days. No antibiotic was used with this procedure.

2.2. Observation of Natural abnormalities in extant horseshoe crabs

We examined 20 adult horseshoe crabs *T. tridentatus* provided by the research institute (GIO). Among these horseshoe crabs, only 5 individuals exhibited signs of natural injury scars. The location of the injuries and the shape of the wound on the horseshoe crabs were observed (Figure 2).

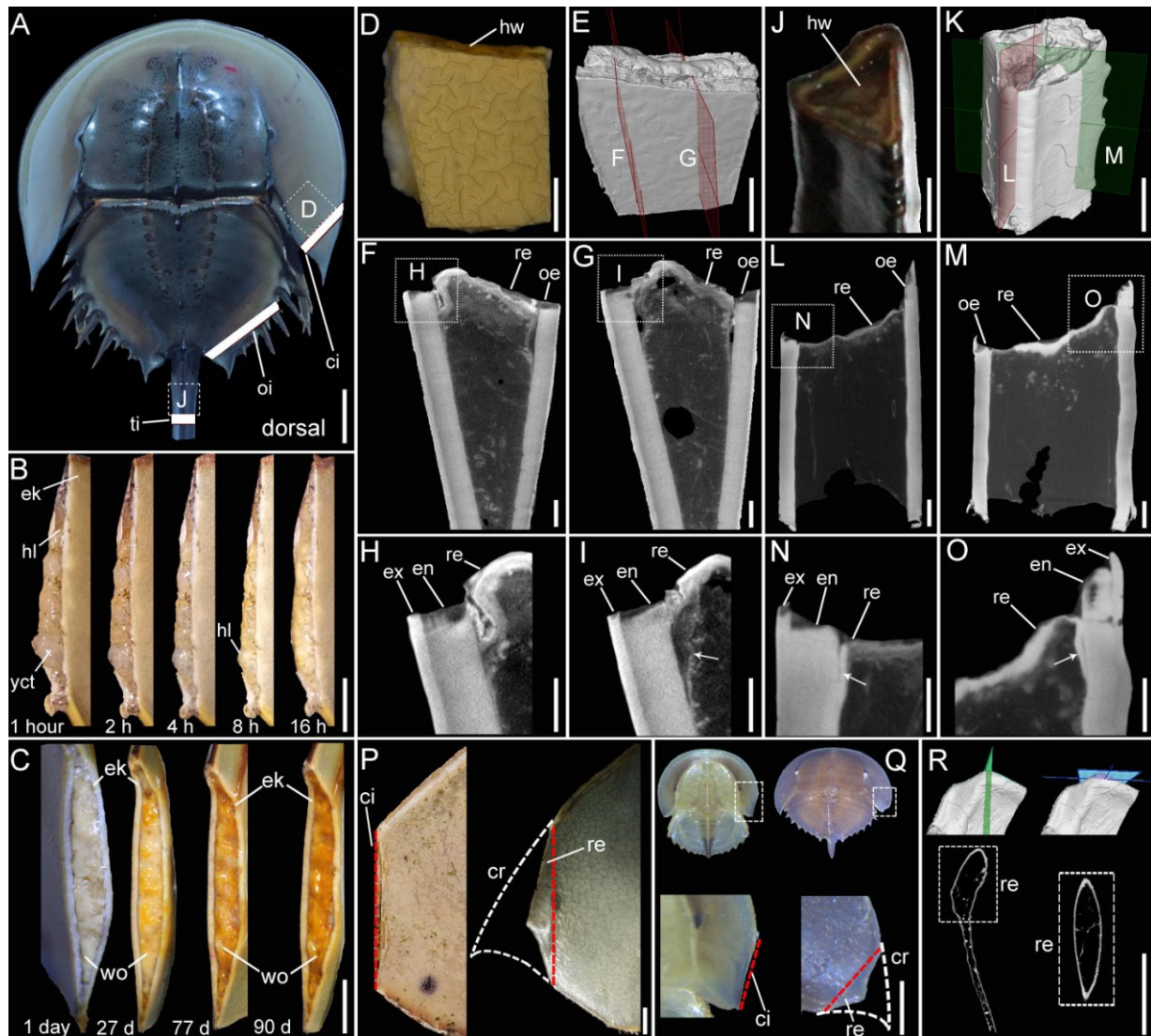


Figure 1. Amputation experiments of extant horseshoe crab *T. tridentatus*. **(A–P)** The quick haemostatic, intermoult cuticle repair and moulting repair in juvenile horseshoe crabs after amputation injuries; **(A)** Dorsal view of three manually injured regions in the present study: cephalothorax, telson and opisthosoma; **(B)** Continuous observations of one cephalothorax injury on a juvenile horseshoe crab after 1, 2, 4, 8 and 16 hours, respectively, complete haemostasis observed within 8 hours after injury; **(C)** A cephalothorax injury on another juvenile after 1, 27, 77 and 90 days; the surface of wound region became harder and the colour changed from light blue to deep brown; moulting was not observed until its death, 179 days after injury; **(D–G)** A cephalothorax injury (119 days after injury); **(J–M)** A telson injury (119 days after injury) examined by light microscopy **(D, J)** and μ -CT 3D reconstruction **(E–I, K–O)**; four μ -CT cross-sections **(F, G, L, M)** randomly selected from both injuries **(E, K)** were magnified (in **H, I, N** and **O**), respectively; a considerable thickness of repair exoskeleton has been produced beneath both side of the adjacent undamaged exoskeleton. The newly regenerated exoskeleton around both injuries were observed on all μ -CT slides, as indicated by arrows (in **N, O**); **(P)** A manual injury on the left cephalothorax of another juvenile horseshoe crab (left) and the repaired cephalothorax with regenerated exoskeletons after moulting (right), 69 days after injury, the dash lines indicate the original cephalothorax boundary (white) and the injured position (red); **(Q–R)** a partially healed cephalothorax injury after the first moulting of the first instar larvae; **(Q)** Comparisons between a newly injured first instar larva (left images) and a new-moulted second instar larva (right images) until 43 days after injury; **(R)** The μ -CT 3D reconstruction of the longitudinal (left) and transverse (right) sections of the same second instar larva (in **(Q)** right), the closed cycle on the lower left indicate the regenerated exoskeletons after moulting. Abbreviations: ci, cephalothorax injury; oi, opisthosoma injury; ti, telson injury; ek, exoskeleton; wo, wound; hw, healed wound; oe, original exoskeleton; re, repair exoskeleton; ex, exocuticle; en, endocuticle; yct, yellow connective tissue; hl, healing layer; cr, cutting region. Scale bars represent: 3 cm **(A)**; 1 cm **(B, C, P)**; 5 mm **(D, E, J, K)**; 1 mm **(F, G, L, M, H, I, N, O, Q, R)**.

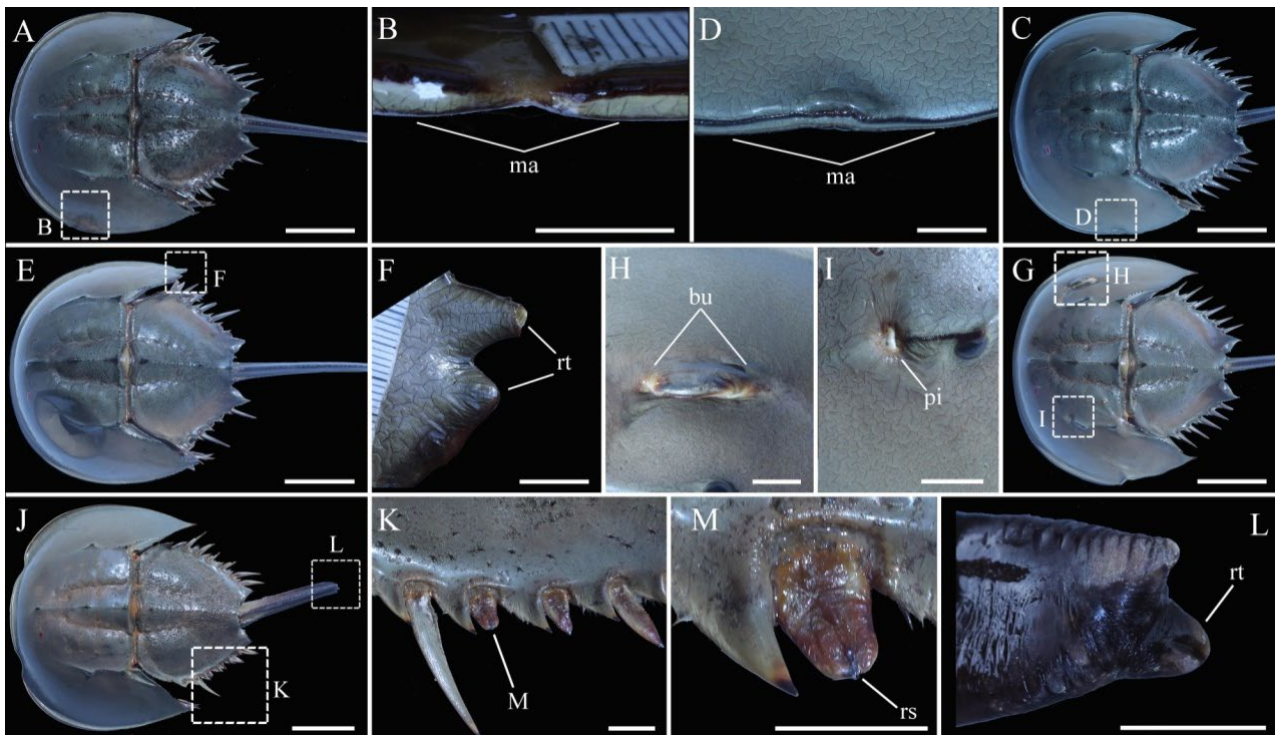


Figure 2. Natural abnormalities on 5 living horseshoe crabs. (A–I) Abnormalities on cephalothorax; (J–K) On opisthosoma; (L) On tail; (A) An individual (XMU-E496) with a marginal abnormality on the left side of cephalothorax, magnified in (B), showing a ‘V’ shaped injury with sealed edge; (C) Another individual (XMU-E483) with a marginal abnormality on the left side of cephalothorax, magnified in (D), showing wrinkling on the edge; (E) An individual (XMU-E497) with a cephalothorax abnormality, magnified in (F), showing two regenerated tips on the right side; (G) An individual (XMU-E491) with two abnormalities on both sides of cephalothorax, magnified images showing a bulge (H) and a pit (I) separately; (J) An individual (XMU-E490) with two abnormalities on the left side of opisthosoma and tail, magnified images showing regenerated spines (K, M) and regenerated tip of tail (L). Abbreviations: bu, bulge; ma, marginal abnormality; pi, pit; rs, regenerated spine; rt, regenerated tip. Scale bars represent: 5 cm (A, C, E, G, J); 1 cm (B, D, F, H, I, K, L, M).

2.3. Amputation experiments of extant horseshoe crab

Twenty-four living juveniles without abnormalities and 440 first-instar larvae of *T. tridentatus* were separately used in five experimental batches, chronologically listed below and in Table S1. The stages of horseshoe crabs were estimated according to the cephalothorax width of each individual. The juveniles were fed with fresh mussel meat at weekly intervals. To avoid potential infection caused by the degradation of excess food, the fresh mussel meat was kept in rearing cases no more than 24 hours. To reduce the risk of water contamination, all first-instar individuals were not fed with any food in rearing duration.

Batch I: 241-day duration. Initially, juvenile horseshoe crabs were used for a preliminary explorative amputation experiments of wound healing lasting for 241 days in the Aquarium Laboratory at the Xiang'an Campus of Xiamen University (XMU) in 2019. Twenty individuals provided by GIO were temporarily reared for two days. After a general examination, 12 individuals were selected and

reared in five plastic cases measuring 800 mm, 500 mm, and 200 mm in length, width, and depth, respectively. The remaining eight individuals were released to the nearest seashore in Xiamen Bay. Seven of the selected individuals were manually injured with an operating scissor in different parts of their anatomy (cephalothorax, opisthosoma, or both) and to differing degrees, while the remaining five individuals were untreated controls (Figure 1A; Table S1). The horseshoe crabs were maintained in sand-filtered seawater (salinity of 28‰–31‰) at room temperature (18–28 °C) with central air-conditioning. The seawater was refreshed daily within the first two weeks and then twice per week later.

Batch II: One-day duration. To imitate the trilobite crawling traces after injury (Figure S1A–I), a juvenile horseshoe crab (14-instar individual) was manually injured and observed for five hours. The whole telson and half of the opisthosoma of this juvenile were removed. The rearing condition of this juvenile is the same as batch I. The horseshoe crab started to crawl two hours after the injury for five minutes. Its crawling traces before and after injury were recorded by photography (Figure S1J,K).

Batches III and IV: 221-day duration. The remaining three experimental batches were conducted in the Zhu'lin Laboratory of GIO for marine aquaculture. Four juvenile horseshoe crabs and 440 newly hatched juveniles (first-instar) were selected for amputation experiments (Table S1). They were originally bred for routine artificial enhancement and release to local sea beaches. The horseshoe crabs were also maintained in sand-filtered seawater (salinity of 32‰) at room temperature (28–34 °C); reared in three glass cases measuring 250 mm, 160 mm, and 180 mm in length, width, and depth, respectively. The batch III was designed for the supplementary observation of the haemostasis process. Four juveniles (body width 10.2–13.7 cm) were manually injured with a scissor on the right margin of cephalothorax. Then the haemostasis around the wound was observed continuously for 48 hours. Images of the wound were taken every 30 minutes. The batch IV was a continuous culture of the four juveniles used in the batch III awaiting their moulting.

Batch V: 124-day duration. The last batch is aimed at studying the regeneration after ecdysis in early horseshoe crab larvae suffering through injuries (Table S1). Four hundred and forty newly hatched first-instar larvae (body width 6.0–7.0 mm) were randomly selected in GIO and reared in ten round plastic basins (diameter of each, 275 mm; depth, 85 mm). Four hundred larvae were manually injured with a scissor uniformly on the right edge of cephalothorax. The remaining 40 larvae were set as untreated controls. When ecdysis was observed, six new second-instar larvae (five injured, one uninjured control) were immediately selected and fixed in 10% formalin for three-dimensional (3D) morphological analyses.

2.4. Photography and light microscopy

Images of the trilobite fossils and horseshoe crabs were directly captured using a Canon EOS 5D camera with a 100 mm macro-lens or a CCD camera connected to a Phenix MC-D500U microscope. All images were combined into figure plates using Adobe Photoshop CS 8.0.1.

2.5. μ -CT 3D analyses

To observe the internal wound morphology during healing in the injured horseshoe crabs, we took six second-instar individuals post-ecdysis and three pieces of tissue from each wound out of four injured juveniles for X-ray micro tomography (μ -CT) using the Zeiss Xradia 520 at Northwest University. Before CT scanning, all tissues were stained with 5% phosphotungstic acid for seven days, then air-dried overnight, and mounted with Parafilm. Up to 1000 μ -CT images (TIFF format) were acquired for each sample, employing beam accelerating voltages of 80 kV–120 kV with a voxel size of 16–18 μ m, respectively. The μ -CT data were processed using Dragonfly 4.0 for 3D volume rendering.

2.6. Comparative measurements for injuries

Three fossils of the trilobite *Eoredlichia intermedia* (*E. intermedia*) from Chengjiang Lagerstatte were re-examined and analyzed by the new diagnoses (SKLELI-0384A, SKLELI-088A, SKLELI-EOM001). Comparative measurements for injuries in the trilobites were performed (Figure 4). The wound/body ratio (WBR, the ratio of the wound length and the anterior-posterior body length, excluding the telson) was calculated to show the injury degree of trilobites (Figure 4A–C) and the horseshoe crabs (Table S2). Revaluation of all trilobites' abnormal scars on the 409 fossils was conducted, integrating the amputation experiment results and listed in the Table S3 as remarks. This literature collection is not exhaustive but provides representative specimens of injured trilobites, including specimens with clear plates, distinct injury characteristics, relatively complete specimens, and specimens that facilitate the statistics of the ratio of injury to body length.

3. Results

The results of amputation experiment indicated that the more severe the injury, the more significant the adverse impact on the horseshoe crab, specifically shortening its survival time. Although no vital organs being damaged, the severity of the injury, which constituted approximately 60% of the body length, was even more fatal for horseshoe crabs (Table S2). Moreover, the incurred damage is also likely to decrease the moulting success rate of horseshoe crabs (Table S1).

3.1. The behavioral manifestations of horseshoe crabs following injury

A severe injury (WBR: 62.2%) was manually performed on a 14-instar horseshoe crab (Table S1, batch II). Following the injury, the horseshoe crab body immediately twisted and subsequently remained immobile for the initial two hours. Two hours after the injury, this horseshoe crab crawled on the sandy bottom for five minutes and left a ploughing trail without footprints (Figure S1J,K). Specifically, the loss of the posterior support (telson/opisthosoma) likely forced the heavy carapace to drag along the substrate like a sled, effectively erasing the footprints made by the remaining anterior appendages. Additionally, noting that the trilobite trace width corresponds to the remaining thorax of the juvenile trilobite (shown in Figure S1A,B).

3.2. The wound repair process of horseshoe crabs

The amputation experiments were conducted using 11 late juveniles (Figure 1A, Table S1, batch I, III) and 440 'trilobite larvae' (Table S1, batch V) of the horseshoe crab

T. tridentatus. The morphological change of wound repair in horseshoe crab consists three stages (haemostasis, cicatrisation before moulting, and further repair after moulting) listed below.

Haemostasis. A haemostatic experiment was conducted on the cephalothoraxes of four late juveniles (WBR, 22.2%–30.0%, Table S1, batch III). At the moment the posterior edge of cephalothorax was cut off, blood immediately oozed out and the wound surrounded by white-floc in half hour (Table S1). Then the original connective tissue was extruded from the wound and a thin pale-yellow film started to form over the wound in one hour (Figure 1B). The wound region was fully covered by the pale-yellow film and the connective tissue was retracted into the old exoskeleton in the 8th hour (Figure 1B), and meanwhile the blood bleeding has ceased.

Cicatrisation (repair before moulting). The healing experiment after haemostasis was carried out on the cephalothorax or opisthosoma of seven late horseshoe crab juveniles (WBR, 20.5%–64.0%, Table S1, batch I). The Cicatrisation repair started from when the wound completely stopped bleeding to the stage before moulting. The colour of the wound changed from light blue to light yellow on the 10th day after injury (Table S1). Then, a dense, pyknotic crust was formed over the surface of the wound on the 27th day (Figure 1C). The crust became harder and changed colour from light blue to deep brown on the 90th day (Figure 1C). The deep brown color of the crust persisted throughout the study; no other visible gross changes were observed (Figure 1C). On the 118th day post-injury, tissues with scars derived from wound regions of two individuals were acquired for μ -CT 3D analyses (Figure 1D,J; Movie S1; Movie S2). The μ -CT 3D reconstruction revealed a thin dense layer (about 0.3mm), appeared to be similar to the normal surface cuticle, covering the wound and sticking on the internal surface of the broken exoskeleton (Figure 1H,I). This newly formed tissue is most likely an exoskeleton, which was located beneath the edge of the remaining exoskeleton, but the sharp outline of the wound remains unchanged (Figure 1D–O). This indicates that at the current stage, no morphological character such as thickening, rounding, or smoothness can be observed.

Regeneration (repair after moulting). Moulting was observed in one injured 14-instar horseshoe juvenile (WBR, 22.2%, Table S1, batch III) and 63 first-instar larvae (WBR, 20.0%–30.0%, see Table S1, batch V). Eleven late juveniles were reared for long-term moulting observation, only one individual moulted on the 68th day post-injury. There is no individual moulted in this set of experiment during 204 rearing days (Table S2). In the moulted juvenile, a small tip regenerated from the remaining exoskeleton (Figure 1P). Additionally, a wrinkle structure appeared on the surface of the newly formed exoskeleton (Figure 1P). The regenerated part was quite small compare to the

normal one on the opposite side. Among the 400 first-instar larvae subjected to manual injury on the cephalothorax, only 63 individuals underwent moulting. The moulting of the first-instar larvae was observed from the 43rd to the 92nd day after injury. The survival rate of the injured first-instar larvae (15.8%) was lower than that of the control group that have not been injured (30.0%). After moulting, the wounds were healed, and a small tip also regenerated for each moulted individual (Figure 1Q), resembling the pattern observed in the late juvenile (Figure 1P). μ -CT examination of the moulted first-instar larvae revealed that the wounds were completely sealed off by the newly developed exoskeleton, which was as thick as the original exoskeleton (Figure 1R; Movie S3; Movie S4). It turns out that the wound became more smooth than before. The sharp wound was no longer visible. To mitigate the risk of water contamination, the larvae were not fed. This might lead to the larvae failing to complete their second moulting during 124 rearing days because of nutritional deficiency.

3.3. Natural abnormalities in extant horseshoe crabs

Natural injury scars resembling those found on trilobites were observed on 5 out of 20 examined individuals of the horseshoe crab *T. tridentatus* (Figure 2). In the central region of the cephalothorax (XMU-E491, Figure 2G), abnormalities manifested as a bulge (Figure 2H) and a pit (Figure 2I). On the anterior edge of the cephalothorax, abnormalities presented as a rounded outline (XMU-E496, Figure 2A,B) or a wrinkled edge (XMU-E483, Figure 2C,D). Extensive regeneration was observed in the marginal area with narrow tips, located on the posterior edge of the cephalothorax (Figure 2E,F), movable spines of the opisthosoma (Figure 2J,K,M), and the distal part of the tail (Figure 2J,L), respectively. An individual (XMU-E497) exhibited a cephalothorax abnormality with two regenerated tips on the right side. Another individual (XMU-E490) had two abnormalities on the left side of the opisthosoma and tail, displaying three regenerated spines (Figure 2K,M) and a regenerated tip of the tail (Figure 2L).

4. Discussion

The repair process includes wound sealing and wound healing, both involve cell movements leading to the establishment of cell contacts between cells (Theopold *et al.* 2004). The observations reported here corroborate those of Bursey (1977) who examined the histological response of the wound repair process in horseshoe crab over a 180-day period. In both sets of observations, an extensive, lamellate repair cuticle was produced after an initial phase of haemostasis, which was dominated by cells. However, no epicuticle repair occurred. This

deficiency has also been reported for the repair cuticles of *Rhodnius* (Lai-Fook 1966), *Tenebrio* (Caveney 1970) and *Carcinus maenas* (Halcrow and Smith 1986) and it might be a general feature of the wound healing response in arthropods (Halcrow and Smith 1986). In this stage, repair cuticle was produced below undamaged cuticle (Fontaine and Lightner 1973). However, whether the difference in the thickness of repaired exoskeleton would be discernible after moulting was not observed in these injured arthropods. Notably, our experiment conducted continuous observations to the moulting stage. And the result demonstrated that exoskeleton growth occurred both prior to and following moulting. However, the repair of the pre-moulting exoskeleton was significantly restricted, and the remarkable regrowth of the lost part occurred solely after moulting. This finding can be utilized for analyzing the malformation of the horseshoe crabs and trilobites.

4.1. Possible causes of abnormalities in horseshoe crabs

The horseshoe crabs considered here have broadly similar malformed morphologies with abnormal trilobites (Figure 2). The deformities in horseshoe crabs are mainly located in the marginal region, while those in the central part are relatively rare, and their types include V-shaped deformities on cephalothoracic margin, bulge or pit on carapace and regenerative small tips. New exoskeletons have formed at the injured margin of the horseshoe crabs, such as the regenerated spines (Figure 2K,M) or tips (Figure 2F,L), as well as the deformed edges (Figure 2B,D), which indicates that the injuries were repaired during the moulting period. These wounds on the edges may have resulted from predation or other forms of mechanical trauma (Das *et al.* 2021). However, the deformity observed in the middle region (Figure 2H,I) lacks a significantly regenerated exoskeleton, making it impossible to determine whether it occurred pre- or post-moulting. In addition to potential mechanical damage, this condition may also be attributed to the influence of parasitic organisms (Groff and Leibovitz 1982; Huggins and Waite 1993; Brianik *et al.* 2023). Therefore, the precise cause and repair mechanism of the deformity in this region remains elusive (Figure 2H,I).

4.2. Comparing with Trilobite injuries

Although some of Radiodonta are considered as sediment sifters and filter feeders (Pates *et al.* 2020; Bicknell *et al.* 2023), at present, the giant radiodont arthropods (*i.e.* *Amplectobelua*, *Anomalocaris*, *Peytoia*, *Lyrarapax*, *Guanshancaris*), the apex predators in the Cambrian and Ordovician (Figure 3), were widely assumed to be the primary natural predator

of trilobites (Nedin 1999; Daley 2013; Fatka *et al.* 2015; Pates *et al.* 2020; Zhang *et al.* 2023). Many other arthropods with raptorial appendages or strong mouth parts from Cambrian Lagerstätten, *i.e.* *Kylinxia*, *Sidynia*, (Zacai *et al.* 2016; Zeng *et al.* 2020), are also candidates. Although the predator groups may be very different (Zacai *et al.* 2016; Pates *et al.* 2020; Zeng *et al.* 2020), the broad mechanics of predation (*e.g.*, crushing, breaking, striking) were likely similar (Bicknell *et al.* 2018). Therefore, the deformities observed in horseshoe crabs and trilobites can be systematically compared. Indeed, horseshoe crabs and trilobites exhibit an extraordinarily high degree of similarity in their deformed structural characteristics (Jell 1989; Bicknell *et al.* 2018). For instance, they all exhibit regenerated spines, U-shaped wound margins, and even display analogous wrinkles on the surface of the newly formed exoskeleton (Figure 2, Figure 4).

As an ancient arthropod, trilobites are presumed to have possessed an open circulatory system similar to that of modern arthropods and must seal the wounds and keep bacteria from entering the hemocoel using efficient clotting systems (Morris and Jenkins 1985). Burling (1917) conducted a comparative analysis of the healing processes in trilobites and crustaceans. While Sinclair (1947) further expanded on these comparisons by examining the healing mechanisms in more detail. Rudkin (1979) hypothesizes that trilobites may have possessed two phases of repair process, analogous to that of extant arthropods. The first phase occurred before moulting that involved haemostasis and wound sealed off. The second phase began at the following pre-ecdysial stage, involved more extensive repair of exoskeleton.

Our experimental results demonstrate indicate that exoskeleton growth around the wound of horseshoe crabs occurs both prior to and following moulting. The newly formed exoskeleton before moulting is only observed on the inner side of the edge of the remaining exoskeleton. This would have been the case also in trilobites by analogy. Consequently, the repair before moulting would have remained imperceptible in compressed preserved fossil records, and may only be discernible in two exceptionally 3D preserved specimens in the literature. In the first case, callus formation on the internal surface of the broken posterior spine was recognized in a 3D preserved pygidium of the trilobite *Tricrepicephalus paracomus* from the Upper Cambrian of USA (Lochman 1941). In the other case, a callus formed on the interior of the broken hypostome in a 3D preserved cephalon of the trilobite *Ceraurinella* recorded from the Ordovician of Canada (Ludvigsen 1977). The absence of callus on the exterior of the hypostome indicates that the wound repair occurred during the intermoult period (Ludvigsen 1977; Rudkin 1979).

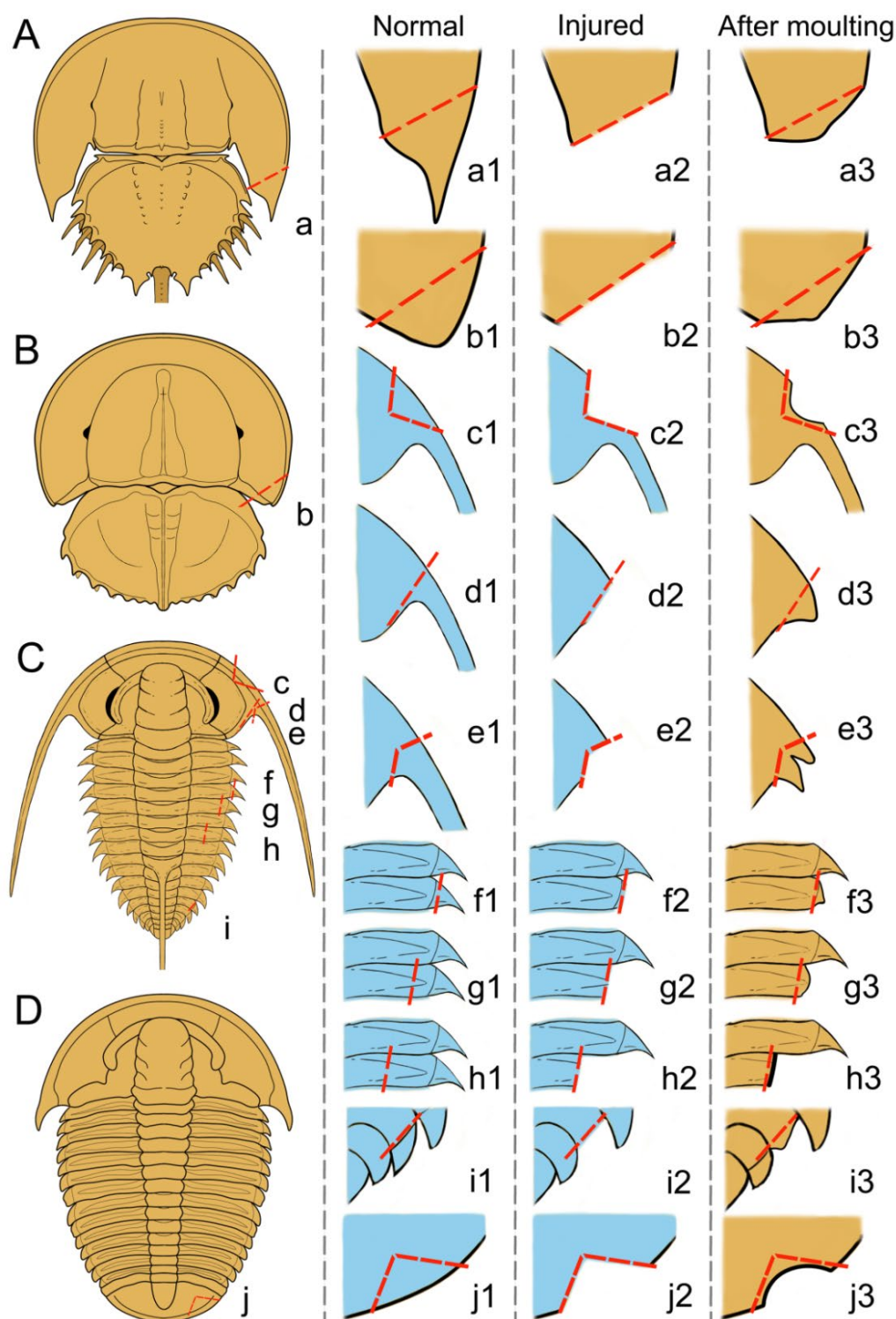


Figure 3. Schematic reconstruction illustrating the morphological change of trilobite wounds in different areas and the degree of injury integrating fossil records and amputation experiments. **(A, B)** Amputation experiment using living juveniles **(A)** and first instar larvae **(B)** of *T. tridentatus*; **(C, D)** A schematic trilobite showing various natural injury localities on cephalon, thorax and pygidium. Data resources: a1–a3, b1–b3, this study; **(D)** Modified after Bicknell and Pates (2020); c3, Modified after Pates and Bicknell (2019); d3, Modified after Hessin (1988); e3, Modified after Snajdr (1990); f3, Modified after Zamora *et al.* (2011); g3, Modified after Capasso and Caramiello (1996); h3, Modified after Hu *et al.* (2013); i3, Modified after McNamara and Tuura (2011); j3, Modified after Snajdr (1980); the remaining reconstructions depicted in blue colour (c1–j1, c2–j2) are inferred through the present integrative analyses.

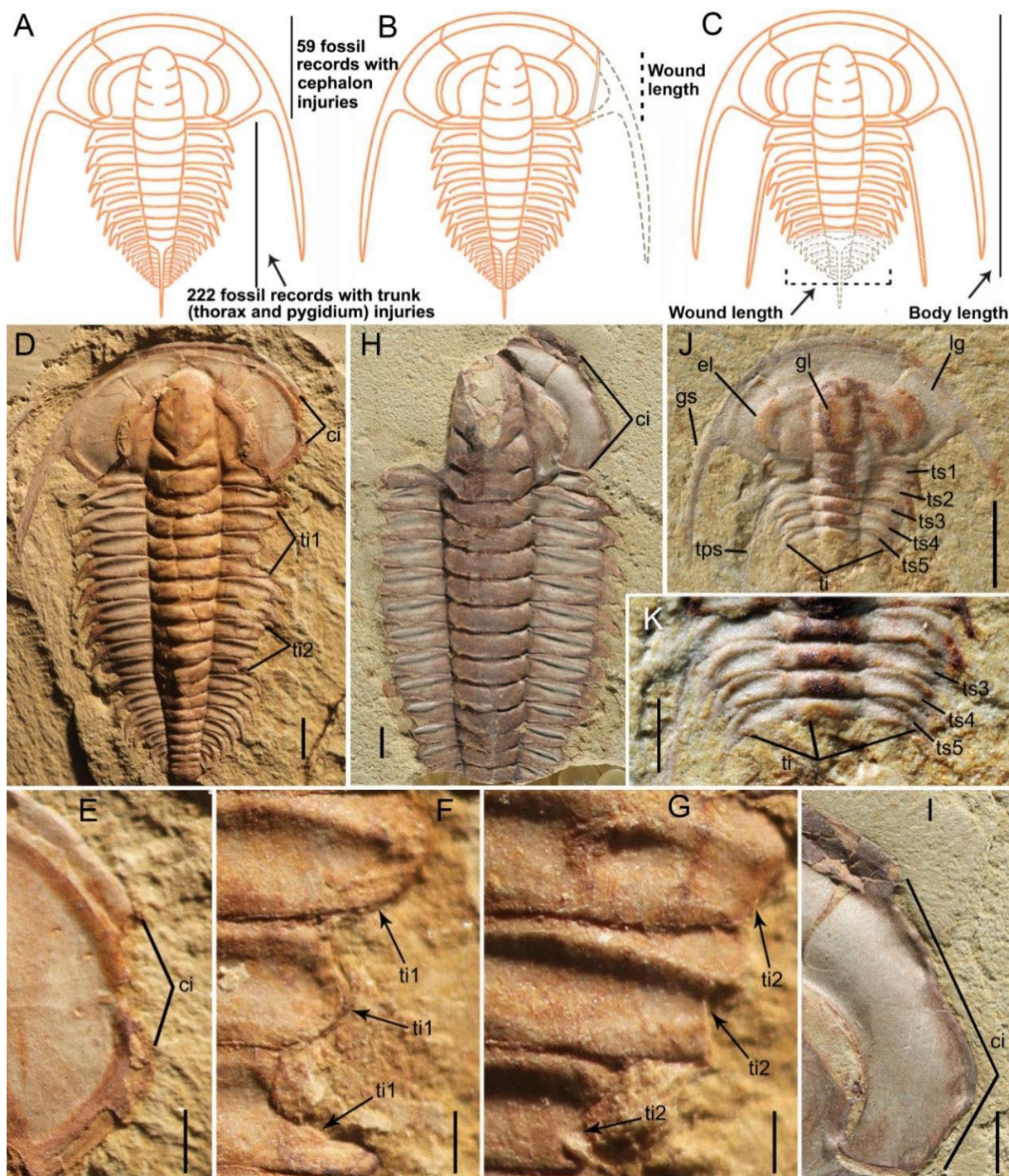


Figure 4. Methodology for assessing the degree of damage. **(A–C)** Schematic drawings upon dorsal views of the trilobite *E. intermedia*, showing the measuring method for body and injury length in fossil trilobites and extant horseshoe crabs used in the present study; **(A)** Statistics for the cephalon and trunk (thorax and pygidium) injuries in trilobites based on 409 trilobite records listed in Table S1; **(B)** An adult trilobite *E. intermedia* with one cephalon injury; **(C)** A juvenile trilobite *E. intermedia* with one thorax injury; **(D–I)** Fossil records of trilobites with injuries from the Cambrian Chengjiang Lagerstätte; **(D–G)** Adult specimen of *E. intermedia* SKLELI-0384A, with one cephalon injury (magnified in E) and two thorax injuries (magnified in F, G), the arrows in F indicate the healed lobate margin where terminally rounded and thickened; **(H–I)** Adult specimen of *E. intermedia* SKLELI-088A, with one healed cephalon injury (magnified in I); **(J)** Juvenile specimen of *E. intermedia* SKLELI-EOM001, missing its last five thoracic segments and pygidium, with sharp margin (magnified in K). Abbreviations: ci, cephalon injury; el, eye lobe; gl, glabella; gs, genal spine; lg, librigena; ti, thorax injury; tps, thoracic pleural spine; ts, thoracic segment. Scale bars represent: 2 mm (D, H, J); 1 mm (E, F, G, I, K).

The present laboratorial results (Figure 1) and natural abnormalities of horseshoe crabs (Figure 2) are heuristic for trilobites. Similar to extant arthropods, trilobites are hypothesized to have undergone a comparable repair process. The assumed morphological change of trilobite wound repair in different regions (cephalon, thorax, and pygidium) and varying degrees of injury for eight representative published trilobite fossils (Snajdr 1980, 1990; Mcnamara and Tuura 2011; Hu *et al.* 2013) has been schematically reconstructed. In conjunction with these results, a new identification criteria for injured trilobites, integrating amputation experiments of extant horseshoe crabs and trilobite fossil records, has been proposed (Figure 3c–j), and the key points of the new diagnostic system are listed below.

(1) The outline of injured trilobite exoskeleton remains unchanged before moulting in compressed preserved fossil records; (2) exoskeletal repair before moulting is almost discernible only on the surface of the wound in 3D preserved fossil records; (3) after moulting, the exoskeleton around the wound changes, appearing as various features of abnormalities, including the regeneration and the deformation of the wound edge; (4) regeneration may occur in limited conditions after moulting, depending on the affected region and trauma severity indices: ① On the blunt edges of the cephalon or pygidium, regeneration cannot occur, but forming a round or smooth outline (Figure 3c3,j3); ② When the injury is located on the marginal area with narrow tips (genal spine, pleural spine and pygidial spine), regeneration is likely to occur (Figure 3d3,e3,f3,g3,i3); ③ However, if the injury is severe, such as losing more than half of the pleural lobe, regeneration of the lost part does not occur, instead forming a scab around the wound (Figure 3h). Regenerative capacity is related to the location and severity of the injury, which exhibits similarities to that observed in extant arthropods, such as the American cockroach (Li *et al.* 2018). This correlation has also been proposed by Pates in trilobites (Pates *et al.* 2017). In another case, the compound-eye lenses of the injured phacopine trilobite were unable to regenerate because of the extensive lesion in the central area of the injury (Schoenemann and Eyzenga 2025). The shape and size of the regenerated parts are genetically determined or controlled by the physical conditions during their establishment (Schoenemann and Eyzenga 2025).

In contrast to previous diagnoses of trilobite injuries that included various specific traits of individual organisms and even particular body segments, the new diagnoses place greater emphasis on the occurrence time and process of morphological change during injury repair. The understanding of the temporal occurrence of exoskeletal alterations solely after moulting is crucial for unambiguously identifying injuries on trilobite fossils.

4.3. The application of new diagnoses to existing injured trilobite records

Three fossils of the trilobite *E. intermedia* from Chengjiang Lagerstätte (Ou *et al.* 2009; Cheng *et al.* 2019) were re-examined and analyzed by the new diagnoses, revealing various types of deformities on the carapace (SKLELI-0384A, SKLELI-088A, SKLELI-EOM001). The location of the injury includes the cephalon, thorax and pygidium (Figure 4D–I). Among these, one adult specimen (Figure 4D) exhibited both sharp (Figure 4G) and smooth wounds distributed along the right side of its body (Figure 4E,F). The sharp wound appears relatively recent, with no evidence of moulting, whereas the smooth wound with thickened edge likely underwent at least one moulting cycle. Consequently, it is plausible that these wounds were not inflicted during the same instar age. This is different from the previous view, which held that these injuries were possibly caused by the same predator. Another specimen (Figure 4H) exhibits a rounded morphology on one side of the cephalon, with a markedly thickened edge (Figure 4I), it must have undergone moulting following the injury. Another juvenile trilobite showed a sharp wound (Figure 4J–K) and one ploughing trace (Figure S1A,B). The sharp wound indicates there was no regeneration (repair after moulting) occurred following the injury. However six aequalate traces surrounded trilobite (Figure S1D–I), similar in appearance with equal width, indicated that it was probably survived after the severe injury and likely underwent the processes of haemostasis and cicatrization.

The new diagnoses have been successfully applied to all 275 listed trilobite fossils exhibiting signs of injury (Figure S3; Table S3). According to these diagnoses, the vast majority of previously documented injured trilobites (95%) exhibited markedly altered exoskeletons of variable individual characteristics, such as arcuate, V-shaped, U-shaped, rounded, thickened, raised, swollen, wrinkled, have all survived at least one moulting after injury.

Only a small proportion of trilobites with sharp edges exhibit reliable evidence of injury. One case is serrated (V-shape to W-shape) wound on several isolated sclerites of the paradoxid trilobite *Mandibulichnus serratus* from the Middle Cambrian of Spain, this type of wound likely caused by a predator or scavenger rather than physical processes (Zamora *et al.* 2011). The sharp V-shaped wound on the cephalon of the trilobite *Olenellus terminatus* from Cambrian Series 2 of USA was also reported as an injury made by a predator (Bicknell *et al.* 2018). These two examples indicate that serrated wounds are more likely to result from predatory activity than damage at the fossil stage. Another study also interrogated this question, and came to similar conclusions (Pates *et al.* 2017). However, other non-serrated wounds are often indistinguishable from taphonomic damage in the absence

of additional diagnostic evidence. Exceptions to this are rare, as exemplified by an *in situ* preserved juvenile trilobite *E. intermedia* mentioned above (Ou *et al.* 2009).

4.4. Recognition biases on Palaeozoic trilobite injuries

In trilobites, repair scars primarily represent evidence of sub-lethal predation (Babcock 1993). Among the 98 injured specimens for which the ratio of injury length could be statistically calculated, the majority of fossil specimens (92%) display relatively minor injuries (WBR < 30%) at the body margin (Figure S3D). This result suggests that wounds exceeding 30% of the body length may have been lethal for trilobites. Similarly, in the experiment conducted on horseshoe crabs, it was observed that the size of fatal wounds was approximately 60% of the body length (Table S2). Although no vital organs were damaged, a large-scale injury may lead to impaired haemostasis and an elevated risk of bacterial infection or other adverse consequences. This indicates that the vast majority of successful predation and fatal damage can't be reliably recognized in the fossil record, in other words that their probability of surviving an attack was minimal (Capasso and Caramiello 1996).

Based on the new diagnoses, a significant number of injured trilobites (95%) have formed new exoskeletons around the wounds, indicating that they likely moulted following injury. (Figure S3F, see remarks for each reviewed fossil in Table S3). But those injuries, occurring during the adult stage are almost unrecognizable or were easily omitted, attributed to their inability to change the shape of the wound through moulting. Because the wound with sharp edge was indistinguishable from post-mortem breakage (Zamora *et al.* 2011). This indicates that a significant number of injured trilobites may have been overlooked, thereby uncovering a novel scientific bias related to the underestimation of trilobite injury frequency. Additionally, exoskeletal repair during moulting could make the injury recognizable by changing the shape of wound in fossil records, however, over several moulting, the healed scars became progressively effaced, and their earlier pronounced configuration gradually disappeared until the overall shape of the affected area was eventually achieved (Snajdr 1981; Pates *et al.* 2017). The repairing process has been illustrated by a series of injured trilobites: (A) Immediately after injury; (B) Rounded spine after initial healing; (C) Regrowth begins, with thin tip of pleural spine; (D) Short spine; (E) Healing complete, spine same length as uninjured spines (see Figure 10 in Pates *et al.* 2017). Therefore, the predation frequency seems to be underestimated because of this completed repair during multiple moulting. Although the wrinkled structures observed in some specimens (Morris and Jenkins 1985) may suggest that trilobites underwent moulting, such features are

only discernible in a limited number of exceptionally well-preserved specimens.

All these above reasons caused by recognition lead to a significant underestimation of predation intensities in the Palaeozoic era. Other biases already acknowledged in relation to taphonomic bias (Babcock 2007), field sampling study bias (Bicknell and Paterson 2017), palaeoclimatic and palaeogeographic influences on predation (Vinn 2017), differing susceptibilities to malformations among various trilobite species (Babcock 2007). These biases highlight the significant incongruity between the intense predator-prey 'arms race' and the infrequent observation of injured trilobites throughout geohistory. Although we are currently unable to deal with these long-established biases, through injury experiments of living arthropods, our understanding of the damage types and repair processes of trilobites will be further enriched. Observing further regeneration of the exoskeleton through a second moult would have provided crucial direct evidence for this study. It is unfortunate that our experiment could not capture a second moulting event of horseshoe crabs probably because of nutritional deficiency. Moreover, the low survival rate (15.8%) of first-instar larvae (Table S1, batch V) might be artificially skewed by starvation and laboratory constraints.

5. Conclusion

We performed an amputation experiment utilizing the extant horseshoe crab as a proxy of trilobites to study the morphological changes in healing and regeneration after injury. The result shows that the newly-formed exoskeleton only regenerates and modifies the shape of the wound after moulting. It suggests that the majority of the trilobite injuries in the fossil record may have been missed, including successful predation events, lethal injuries, and damage incurred during the adult stage, as the sharp wounds were indistinguishable from post-mortem breakage. This experiment confirmed the influence of recognition biases leading to the underestimation of trilobite injury frequency.

Data availability statement

The supplementary files are provided with this article. [Supplementary Figure S1](#)—Comparison of crawling traces of a trilobite and a horseshoe crab; [Supplementary Figure S2](#)—An artistic reconstruction of the imaginary Cambrian predator-prey 'arms race' scene *in situ* for the injured trilobites; [Supplementary Figure S3](#)—Statistics on 409 selected abnormal trilobite fossils ranging from Cambrian to Carboniferous; [Supplementary Table S1](#)—Amputation experiments using late juveniles and first larvae of the horseshoe crab *T. tridentatus*; [Supplementary Table S2](#)—Statistics for eight horseshoe crab juveniles during the healing experiment (Batch I); [Supplementary Table S3](#)—Comprehensive review and reevaluation of 409 Palaeozoic trilobite records with abnormalities selected for statistics in [Figure S3](#); [Supplementary Movie 1](#)— μ -CT 3D construction video showing the cephalothorax region of a late juvenile of the horseshoe crab before moulting (119 days after injury); [Supplementary Movie 2](#)— μ -CT 3D construction video showing the telson region of a late juvenile of the horseshoe crab before moulting (119 days after injury); [Supplementary Movie 3](#)— μ -CT 3D construction video showing the cephalothorax region of a first instar larva of the horseshoe crab before moulting (2 hours

after injury); [Supplementary Movie 4](#)— μ -CT 3D construction video showing the cephalothorax region of a second instar larva of the horseshoe crab after moulting (43 days after injury).

Declaration of generative AI and AI-assisted technologies

The authors did not use generative AI or AI-assisted technologies in the writing of this manuscript, including all figures presented in the text.

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Authors' contribution

Conceptualization, J.H.; methodology, J.H. and X.Y.; software, J.S.; validation, J.H. and X.Y.; formal analysis, J.S. and X.Y.; investigation, M.C. and X.Y.; resources, R.C.; data curation, S.L. and H.L.; writing—original draft preparation, X.Y.; writing—review and editing, X.Y.; visualization, X.L.; supervision, J.H.; project administration, J.H.; funding acquisition, J.H. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

Jian Han holds the position of Executive Editor for *Continent and Life Evolution* and has not peer reviewed or made any editorial decisions for this paper. Ruifang Chen, Haijuan Liu and Shicai Li are employees of Guangxi Institute of Oceanology Co., LTD. The authors declare that this affiliation does not influence the design, analysis, interpretation, or presentation of the results reported in this manuscript. The authors declare no other conflicts of interest.

Ethical statement

The research protocol related to laboratorial experiments using living juveniles and larvae of the horseshoe crab *T. tridentatus* was approved by the Committee for the Care and Use of Laboratory Animal Ethics of Northwest University, China (License number, NWU-AWC-20190104H) on January 3rd, 2019. All horseshoe crabs used here were supplied by the Guangxi Institute of Oceanology in accordance with a license for artificial propagations and scientific research in *T. tridentatus*. The license was approved by the Department of Agriculture and Rural Affairs of Guangxi Zhuang Autonomous (License number, Guiyu 201800695; term of validity, April 17th, 2018–December 4th, 2021).

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