A collection of horseshoe crabs (Chelicerata: Xiphosura) in the National Museum, Prague (Czech Republic) and a review of their immunological importance

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Abstract. The zoological collection of the National Museum, Prague (NMP) contains spirit (juvenile) as well as dry (mostly adult) specimens of horseshoe crabs (Xiphosura). Living horseshoe crabs are of immunological importance due to clotting agents present in their hemolymph. Here we summarize basic data about the mechanism of the immune system of these marine animals and its use in practice – the *Limulus* Amebocyte Lysate test – including the laboratory assays and handling with the animals. In the NMP collection, 82 specimens (16 dry and 66 spirit) of all four currently recognised living species of horseshoe crabs are present. They were collected in Indonesia, USA and Vietnam in 1872–1998; *Limulus polyphemus* from the USA is the most numerous species in the NMP. The collection contains no type specimens but three historical mounted ontogenetic series are present. The largest part of the horseshoe crab collection is 55 spirit specimens from the collection of Václav Frič (1839–1916) whose preparations were intended mostly for educational purposes.

Keywords: identification key, LAL test, pharmacology, Václav Frič, Xiphosurida, zoological collection

Horseshoe crabs are marine bottom-dwellers, growing up to 85 cm long. Their prosoma is covered with a single unjointed carapace bearing two compound eyes. Research on the compound eyes of horseshoe crabs has led to a better understanding of human vision and based on their results, Ragnar Granit, Haldan Keffer Hartline and George Wald were awarded the Nobel Prize in Physiology or Medicine in 1967 (Nobel Media AB 2014). The opisthosoma bears paired movable lateral spins and an unpaired telson. There are four Recent species described: *Limulus polyphemus* (Linnaeus, 1758) from the North American East coast, and *Carcinoscorpius rotundicauda* (Latreille, 1802), *Tachypleus tridentatus* (Leach, 1819) and *Tachypleus gigas* (O. F. Müller, 1785) all from South-East Asia. All four species are similar in terms of ecology, life history and serology. They burrow through the surface layers of muddy substrate and ingest smaller animals or scavenge. The so-called trilobite larvae hatch from the eggs; the horseshoe crabs reaches maturity after 9–12 years and the life span may be up to 19 years. Males differ from females in having modified the first two pairs of walking legs into claspers used during mating. Horseshoe crabs occupy a crucial place in the food chain in coastal ecosystem – molluscs, crustaceans, fish, leopard sharks, eels, migratory shorebirds and sea turtles prey on horseshoe crabs during different stages of their life cycle (Shuster 1982, Keinath et al. 1987). Other features of their biology and ecology can be found in Sekiguchi (1988), Shuster et al. (2003) and Tanacredi et al. (2009).

History of the taxonomy of horseshoe crabs was summarized in Dunlop et al. (2012), their phylogenetic relationships were revealed by Obst et al. (2012) and their current taxonomical position was revised by Lamsdell (2013) as follows:
Subphylum: Chelicerata Heymons, 1901
Class: Xiphosura Latreille, 1802 (syn. Merostomata Dana, 1852)
Order: Xiphosurida Latreille, 1802
Family: Limulidae Leach, 1819

The diploid chromosome numbers (2n) of horseshoe crabs were published by Iwasaki et al. (1988). Recently, mitochondrial genome sizes (MGS) and a genome adenine-thymine base ratio (AT) of horseshoe crabs have been studied (Lavrov et al. 2000, Baek et al. 2014) resulting in the following genetic characteristics – *L. polyphemus*: 2n = 52, MGS = 14985 bp, AT = 67.6%; *C. rotundicauda*: 2n = 32, MGS = 15033 bp, AT = 73.8%, *T. tridentatus*: 2n = 26, MGS = 15006 bp, AT = 74.0%; *T. gigas*: 2n = 28, MGS and AT yet unknown. In addition to this, horseshoe crabs are of great clinical importance due to clotting agents present in their hemolymph as summarized below.

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Currently, many museums are publishing catalogues of their collections (e.g. Jiroušková et al. 2011, Chiarle et al. 2012, Dunlop et al. 2012, 2014, Kielhorn et al. 2012, Mlíkovský et al. 2013, Seiter & Hörweg 2013). Thus, the aim of this paper is to summarize the practical use of horseshoe crabs and to provide further information about the zoological collections of the National Museum in Prague.

The American horseshoe crab – a guardian of public health

The study of haematology using the horseshoe crab as a model is possible due to large blood cells (amebocytes) and led to discovery of the *Limulus* Amebocyte Lysate test (LAL test) by Bang & Forst (1953). They observed that infection by gram negative bacteria caused intravascular coagulation in the horseshoe crab. Coagulation is activated by the cascade of enzymes located in the amebocytes and is triggered by lipopolysaccharide (LPS, endotoxin) in the gram negative bacterial cell walls (Levin & Bang 1964a, 1964b, 1968). The catalytic nature of each activated enzyme in the coagulation cascade serves in turn to amplify the next step, resulting in a high sensitivity of LAL to LPS (Iwanaga 2007).

Content of microorganisms in the seawater can reach up to 10^6 bacteria/ml and 10^9 virus/ml of seawater (Ammerman et al. 1984). Therefore, the effective and robust innate immune system of horseshoe crabs is a prerequisite for their survival in this environment (Menzel et al. 2002, Tincu & Taylor 2004). It has been suggested that the clot formed through the activation of the cascades captures and immobilizes invading microorganisms; clot formation is triggered not only by an endotoxin released by Gram negative bacteria but also by (1,3)-β-D-glucan (Morita et al. 1981) which is mainly present in the cell walls of yeast and other fungi (Iwanaga 1993, 2002, Muta et al. 1995). Moreover, the clot formed as a result of activation by endotoxin or glucans provides wound control by preventing bleeding and forming a physical barrier against additional infection entry (John et al. 2010, Armstrong et al. 2013). Thus, the *Limulus* clotting system is thought to be critical for both haemostasis and the biological defence of this animal (Iwanaga 2007). This gelation reaction of *Limulus* amebocyte lysate has been widely employed as a rapid and simple method for endotoxin testing. How then do horseshoe crabs protect public health?

Endotoxin (LPS) is a part of the outer membrane of Gram-negative bacteria and is released during lysis of the cell or cell division. Most problems for the pharmaceutical industry are caused by non-pathogenic Gram-negative bacteria amply represented in aquatic environments. The high occurrence of endotoxin cannot be excluded even in sterile products, since endotoxin is able to withstand steam sterilization. Mild Gram-negative bacterial infections can trigger a pyrogenic response. The presence of endotoxin in the blood stream can cause fever, inflammation, and frequently irreversible shock (Joiner et al. 2002). Since humans are sensitive to minute amounts of endotoxin, the importance of testing for endotoxin is apparent.

In the early 1970s, a newer pyrogen testing technique using horseshoe crabs' blue copper-based blood was developed. The LAL test would be 100 times more sensitive than the rabbit testing methods used before (Novitsky 1984). Clot formation is initiated by pico- to nanograms of bacterial endotoxin (Mikkelsen 1988). Because of its superior reliability and simplicity (the test takes only one hour), the LAL test became an invaluable tool for the pharmaceutical industry. Every drug and medical device certified by the Food and Drug Administration must be tested by a LAL test (Walls & Berkson 2000).

The LAL test is employed to ensure that pharmaceutical products are endotoxin-free including bulk lot release testing, final product release testing and raw materials testing (Rudloe 1983). Anyone who has ever encountered intravenous fluids, vaccines or surgical implants has been protected against contact with bacterial endotoxin by the blue blood of a horseshoe crab. The LAL test is used not only to determinate harmful levels of endotoxin in pharmaceutical products, but is also the backbone of controlling endotoxin in both the process and equipment involved in producing pharmaceuticals and of monitoring high purity water used as a prime source. Furthermore, the LAL test is the method of choice for researchers examining the clinical or the environmental effects of endotoxin (Walls et al. 2002).

Currently, three principal LAL test methods exist; the gel clot, turbidimetric and chromogenic methods. The latter two are referred to as the photometric method for they require an optical reader. The gel clot assay is the simplest method of determining the level of bacterial endotoxin. In the assay, equal volumes of LAL reagents are mixed with the

tested sample and clot formation is observed. At the end of the incubation period the tube containing the mixture of the sample and LAL is inverted. If a gel has formed and remains intact in the bottom of the reaction tube after an inversion of 180 degrees (Fig. 1), the test is positive. A positive test indicates that the concentration of endotoxin in the tube is greater than or equal to the sensitivity of LAL (Associates of Cape Cod Inc. 2007a).

Both photometric methods require a standard curve to determine the endotoxin level in the sample. The chromogenic assay is based on replacing the natural substrate, coagulogen, by a chromogenic substrate. The chromogenic substrate is cleaved by the serine protease coagulose activated by endotoxin, and then the chromophore is released and is measured by spectrophotometry (Associates of Cape Cod Inc. 2011). The turbidimetric method is analogous to the chromogenic method, but the turbidity is monitored (Joiner et al. 2002).

In the blood of the horseshoe crab, other compounds of biomedical interest have also been discovered. LAL is used for detecting 1,3-β,D-glucans e.g. in pharmaceutical products or in a test for fungal infection (Obadasi et al. 2004, Associates of Cape Cod Inc. 2007b). Furthermore, an endotoxin-neutralizing protein which has potential as an antibiotic as well as an alternative endotoxin assay, and a number of other proteins that show anti-viral and anti-cancer activity are being explored (Valespi et al. 2000, Andrä et al. 2004, Tincu & Taylor 2004).

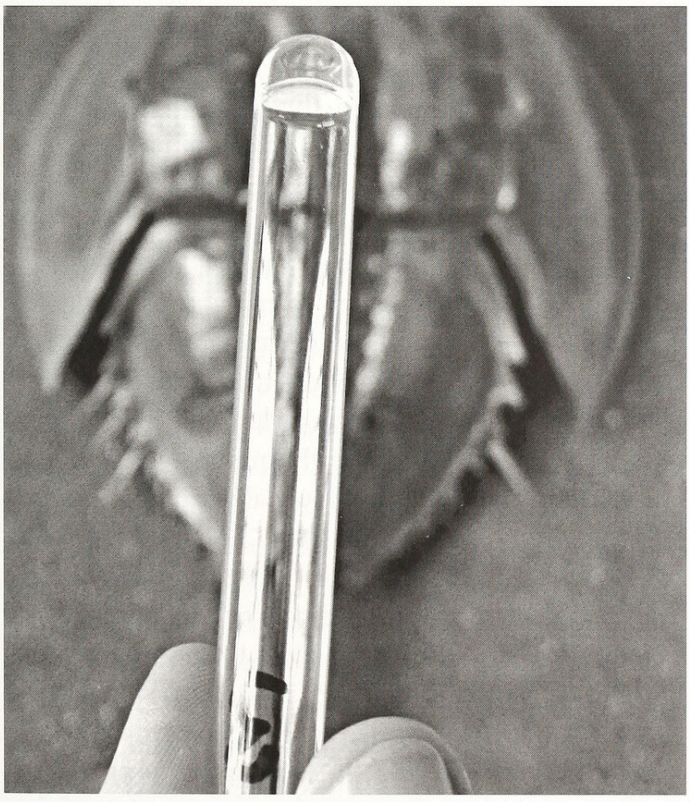


Fig. 1: The positive result of a gel clot assay (photograph provided by Biogenix, s. r. o., reprinted with permission)

Fig. 2: Bleeding of horseshoe crabs (photograph provided by Associates of Cape Cod Inc., reprinted with permission)

And how is the blood of the horseshoe crab obtained? Adult horseshoe crabs are collected by trawlers or by hand-harvest and transported to the lab of a biomedical company, where they are washed and placed on a rack. Horseshoe crabs are bled from pericardium with a large gauge needle – up to 30% of the animal's blood is removed (Fig. 2). Within 72 hours, the bled horseshoe crabs are returned to the place of capture and released alive (ASMFC 1998, Leschen & Correia 2010). Their blood volume restores in about a week. The amebocytes regenerate at a slower rate, requiring up to four months before cell counts equal to those obtained prior to bleeding (Novitsky 1984).

Mortality of horseshoe crabs after the bleeding process was found to be 3-15 % (Walls & Berkson 2000). There are currently five biomedical companies producing LAL in the United States. Each of them has unique bleeding methods, method of capture, distance and method of travel to bleeding lab, holding time and conditions, and methods of return most appropriate to their own setting and situation. Thus, the impact of the blood extraction processes on survival of the horseshoe crabs varies between operations (Walls et al. 2002).

The blood of horseshoe crabs can be extracted without killing the animals, but nowadays attention is paid to long-term injury caused by the bleeding process. Recent studies denote that the biomedical bleeding process potentially led to several sub-lethal behavioural and physiological changes. The most obvious behavioural effects are immediate decreases in walking speed and latent reductions in both overall activity and the expression of tidal rhythms. The greatest impact of bleeding on *Limulus* physiology is an immediate and sustained decline in hemocyanin concentrations (Anderson et al. 2013).

Horseshoe crab blood is not only a backbone of pharmaceutical industry, but it is also big business. On the world market, a quart of horseshoe crab blood has a price tag estimated at $15000, leading to overall revenues from the LAL industry estimated at U.S. $50 million per year. According to the Atlantic States Marine Fisheries Commission, that $50 million dollar industry requires the blood of approximately 500,000 horseshoe crabs (ASMFC 2013).

Fortunately, companies producing LAL realize that a stable population of horseshoe crab is essential not only for the pharmaceutical industry but also for survival of other marine animals that have a symbiotic relationship with the horseshoe crab. The LAL industry has taken steps to make the LAL test synthetically or to find methods to improve the sensitivity of LAL, which would eliminate the use of live horseshoe crabs for the LAL reagent (Thorne et al. 2010). With growing concern over declining populations, it is obvious that it will be a challenge to ensure that horseshoe crabs manage to fulfil all these diverse needs and at the same time to ensure their sustainable population for the future.

The situation is bit different in Asian horseshoe crabs. The *Tachypleus* Amebocyte Lysate (TAL) is derived from the two *Tachypleus* species – *T. tridentatus* and *T. gigas*. Nevertheless, only two species, *L. polyphemus* and *T. tridentatus* are mentioned in the European Pharmacopeia, the United States Pharmacopeia and in the Japanese Pharmacopeia. In Asia, TAL is manufactured in China and Japan (Wang et al. 2007). However, most of the animals used come from areas in Southeast Asia where harvesting regulations have not been established or enforced and less is known regarding their horseshoe crab collection and handling practices. It is believed that most horseshoe crabs die post-bleeding, some as bait, some as food, and some due to the bleeding process itself.

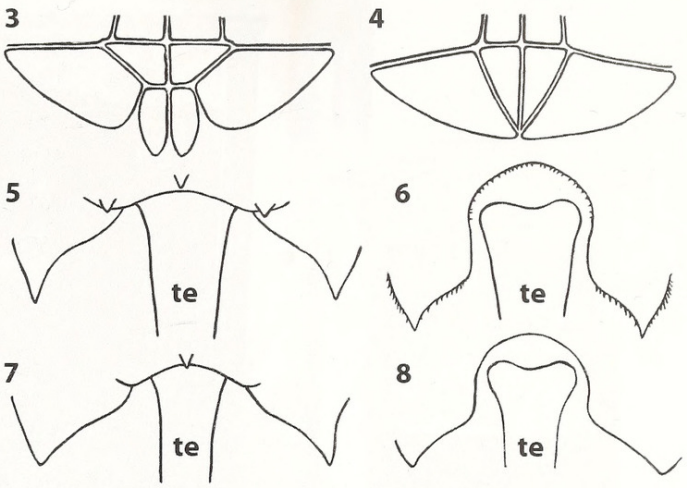
Material and methods

Methods for curating the horseshoe crab collection followed those of Dunlop et al. (2012). Specimens were determined by the key provided below that was composed based on characteristics published by Yamasaki (1988), Shuster & Anderson (2003) and Dunlop et al. (2012). Current nomenclature and the Life Science Identifier numbers (lsid) were adopted from WoRMS (2014). The items belonging to one species are sorted chronologically from the oldest to the newest one. Data for each item are arranged as follows: inventory number under the acronym NMP (National Museum Prague), number of specimens and their sex (type of preparation), name of the collector, date of collection, locality – the current name of the locality was adopted from NGA (2014); note if any. In dry material, total length (TL) and carapace width (CW) is provided. If the telson is missing or broken, only CW is noted.

Identification key for living species

1) A pair of finger-like projections on the genital operculum (Fig. 3); the first entapophyseal pit on opisthosoma triangulate (left ┌, right ┐); telson shorter than the body; distributed in the western Atlantic along the eastern seaboard of North-Central America ..... *Limulus polyphemus*
– No finger-like projections on the genital operculum (Fig. 4); the first entapophyseal pit on opisthosoma elongate (I); telson at least as long as the body; distributed in the Indo-Pacific waters of South-East Asia ..... Tachypleinae (2)
2) Telson longer than the body, smooth, rounded in profile (without any ventral groove or dorsal keel); all opisthosomal marginal spines short (Fig. 12); total length up to 35 cm in males and 40 cm in females ..... *Carcinoscorpius rotundicauda*
– Telson as long as the body, triangular in profile (usually with a ventral groove and dorsal keel); the first three (in females) or all (in males) opisthosomal marginal spines long; usually larger animals ..... *Tachypleus* (3)
3) Total length 45-70 cm in males and 55-85 cm in females; mid-dorsal part of opisthosomal posterior margin with three short immovable spines pointing posteriorly (Fig. 5); anal angles (from ventral view) with thorn-like spines on both outer lateral margins (Fig. 6); males with a pair of strong indentations to the anterior rim of the prosoma (Fig. 13) ..... *T. tridentatus*

– Total length 25-40 cm in males and 25-50 cm in females; mid-dorsal part of opisthosomal posterior margin with one short immovable spine pointed posteriorly and with no spines on either side (Fig. 7); anal angles (from ventral view) usually with smooth outer lateral margins (although they rarely have small spines on the margin) (Fig. 8); males with smooth margins of the anterior rim of the prosoma (Fig. 14) ..... *T. gigas*



Figs 3-8: Diagnostic characters of horseshoe crabs. **3:** *Limulus polyphemus*, a pair of finger-like projections on the genital operculum; **4:** Asian horseshoe crabs, no finger-like projections on the genital operculum; **5:** *Tachypleus tridentatus*, mid-dorsal part of opisthosomal posterior margin with three short immovable spines pointed posteriorly; **6:** *T. tridentatus*, anal angles (from ventral view) with thorn-like spines on both outer lateral margins; **7:** *Tachypleus gigas*, mid-dorsal part of opisthosomal posterior margin with one short immovable spine pointed posteriorly and with no spines on either side; **8:** *T. gigas*, anal angles (from ventral view) with smooth outer lateral margins; te = telson

Systematic list

Subfamily: Limulinae Leach, 1819

Genus: *Limulus* O. F. Müller, 1785

Limulus polyphemus (Linnaeus, 1758)

urn:lsid:marinespecies.org:taxname:150514

NMP P6E-2460, 1 ♂ (dry material, TL = 38 cm, CW = 20 cm), collected by Dr. Palacký in 1872 in North America.

NMP 19/1960/2564, 6 juveniles (spirit material), collected by an unknown collector in 1886 in Woods Hole (41°31'25"N 70°40'20"W), USA; ex. coll. V. Frič.

NMP 19/1960/2745, 30 juveniles (spirit material), collected by an unknown collector in 1886 in Woods Hole (41°31'25"N 70°40'20"W), USA; ex. coll. V. Frič.

NMP P6E-3903, mounted ontogenetic series containing 18 eggs and 4 juveniles (spirit material), donated by A. S. Packard to J. Barrande from whose inheritance it arrived in the NMP in 1894; Fig. 9.

NMP P6E-2462, mounted ontogenetic series containing 4 eggs and 6 juveniles (spirit material), collected by an unknown collector on 24 September 1896 in New York, Long Island (40°37'00"N 73°50'20"W), USA; Fig. 10.

NMP 19/1960/2580, 7 juveniles (spirit material), collected by Brimley Bros Raleigh in February 1904 in USA; ex. coll. V. Frič.

NMP 19/1960/2905, 1 juvenile (spirit material), collected by an unknown collector on 25 January 1913 in Woods Hole (41°31'25"N 70°40'20"W), USA; ex. coll. V. Frič.

NMP P6E-2461, 1 ♀ (dry material, TL = 56 cm, CW = 27 cm), collected by an unknown collector in 1913 in North America; ex. coll. V. Frič.

NMP 19/1960/2280, 1 juvenile (spirit material), unknown origin; ex. coll. V. Frič.

NMP 19/1960/2281, 1 trilobite larva (spirit material), unknown origin; ex. coll. V. Frič.

NMP 19/1960/3100, mounted ontogenetic series containing 4 eggs and 9 juveniles (spirit material), unknown origin; ex. coll. V. Frič; Fig. 11.

NMP P6d-8/2003, 1 malformed ♀ with broken telson (dry material, CW = 29 cm) and 1 ♂ (dry material, TL = 39 cm, CW = 19 cm), unknown origin.

NMP P6d-254/2003, 1 female carapace (dry material, TL = 61 cm, CW = 29 cm), collected by Mr. Morawitz in 1957 in New York, Long Island (40°37'00"N 73°50'20"W), USA.

NMP P6E-2794, 1 ♀ and 2 juveniles (dry material, TL = 37, 4 and 3 cm, CW = 18, 2 and 2 cm), collected by D. Collins in 1968 in Naples, Florida (26°08'30"N 81°48'30"W), USA.

Other material: 1 ♂ in poor condition (dry material, TL = 38 cm, CW = 21 cm) and 2 damaged (probably female and male) carapaces (dry material, TL = ? and 39 cm, CW = 25 and 19 cm), unknown origin; packed in a newspaper "Národní politika" [National politics] from 22 March 1929.

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Fig. 9: Mounted ontogenetic series of *Limulus polyphemus* (NMP P6E-3903), inheritance of J. Barrande
Fig. 10: Mounted ontogenetic series of *Limulus polyphemus* (NMP P6E-2462), from the former zoological exhibition

Subfamily: Tachypleinae Pocock, 1902
Genus: *Carcinoscorpius* Pocock, 1902

Carcinoscorpius rotundicauda (Latreille, 1802)
urn:lsid:marinespecies.org:taxname:238267
NMP P6E-3085, 1 ♂ (dry material, TL = 33 cm, CW = 15 cm), collected by J. Wünsch in May 1973 in Tuân Châu, Vịnh Hạ Long (20°55'40"N 106°59'40"E), VIETNAM; Fig. 12.

Genus: *Tachypleus* Leach, 1819

Tachypleus tridentatus (Leach, 1819)
urn:lsid:marinespecies.org:taxname:238270
NMP P6E-2838, 1 ♂ (dry material, TL = 60 cm, CW = 28 cm), collected by Dr. Hložánek in 1988 in VIETNAM; Fig. 13.

NMP P6E-3086, 1 young ♀ (dry material, TL = 61 cm, CW = 32 cm), collected by K. Vopařil in 1998 in VIETNAM; originally labelled as "*Limulus grandis*".

Tachypleus gigas (O. F. Müller, 1785)
urn:lsid:marinespecies.org:taxname:238271
NMP P6E-3904, 1 juvenile (spirit material), collected by an unknown collector in 1898 in Maluku (3°50'S 129°50'E), INDONESIA; originally labelled as *Limulus moluccanus*.

NMP P6E-3120, 1 ♀ (dry material, TL = 40 cm, CW = 19 cm), collected by Dr. Jerman on 17 December 1933 in Gunung Pantaicarmin, Sumatera Barat (1°22'60"S 100°34'30"E), INDONESIA.

NMP P6E-3121, 1 ♂ (dry material, TL = 31 cm, CW = 15 cm), collected by Dr. Jerman on 11 No-

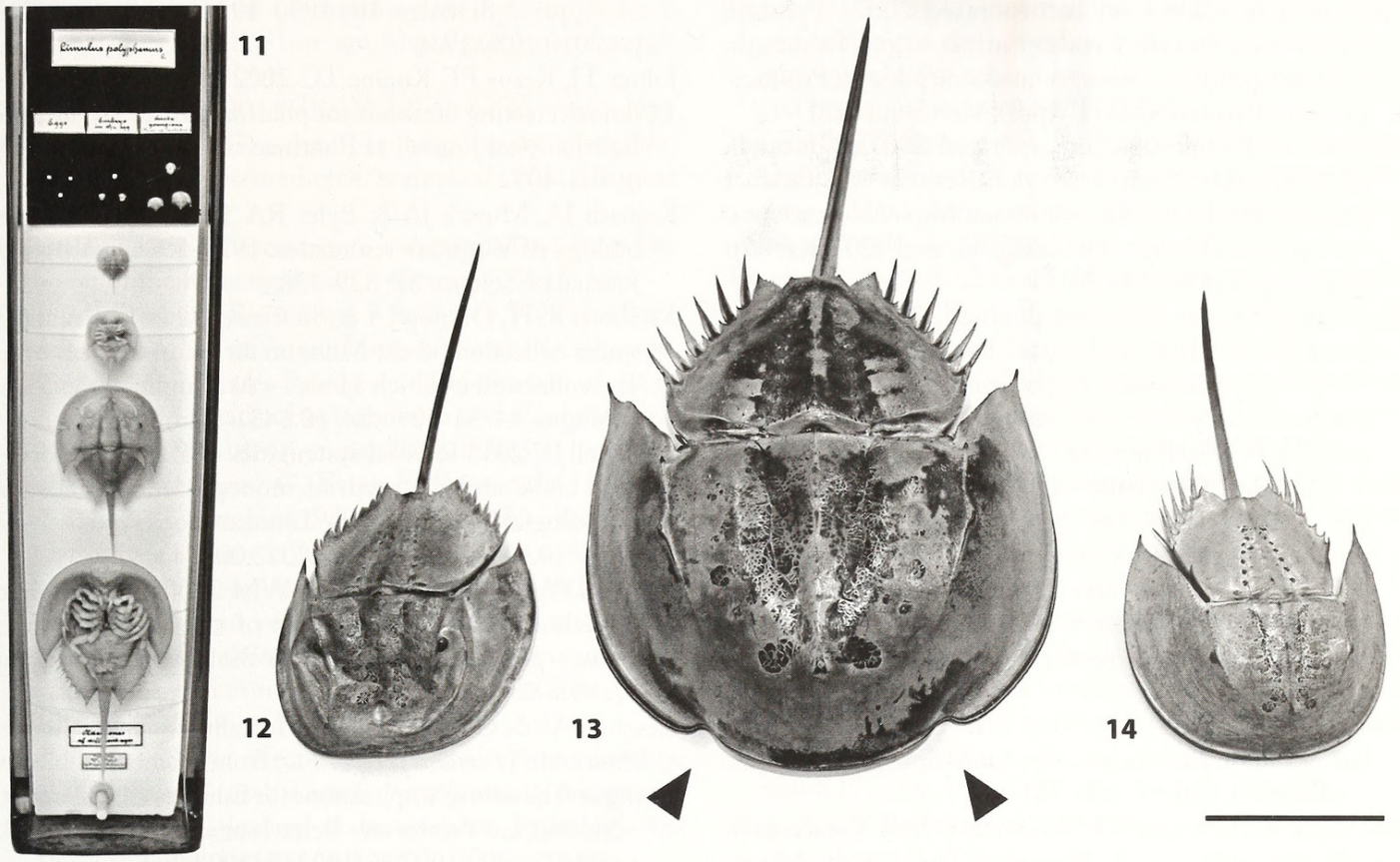

Fig. 11: Mounted ontogenetic series of *Limulus polyphemus* (NMP 19/1960/3100), ex. coll. V. Frič
Figs 12-14: Males of horseshoe crabs in relation to one another. **12:** *Carcinoscorpius rotundicauda* (NMP P6E-3085), note short opisthosomal movable marginal spines; **13:** *Tachypleus tridentatus* (NMP P6E-2838), note a pair of strong indentations to the anterior rim of the prosoma (arrowheads); **14:** *Tachypleus gigas* (NMP P6E-3121), note smooth margins of the anterior rim of the prosoma; scale bar = 10 cm

vember 1934 in Gunung Pantaicarmin, Sumatera Barat (1°22'60"S 100°34'30"E), INDONESIA; Fig. 14.

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