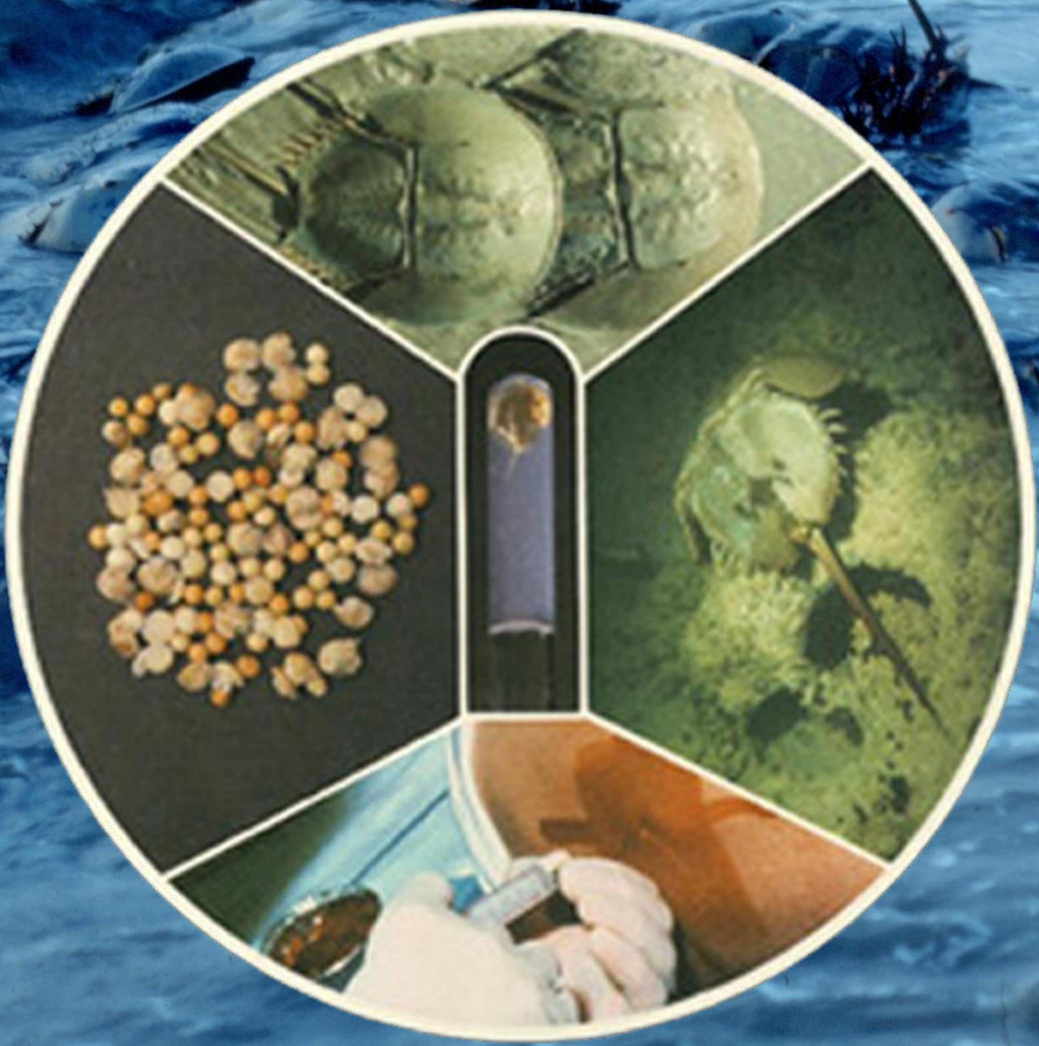
The Secret in the Blue Blood

by Tom Mikkelsen



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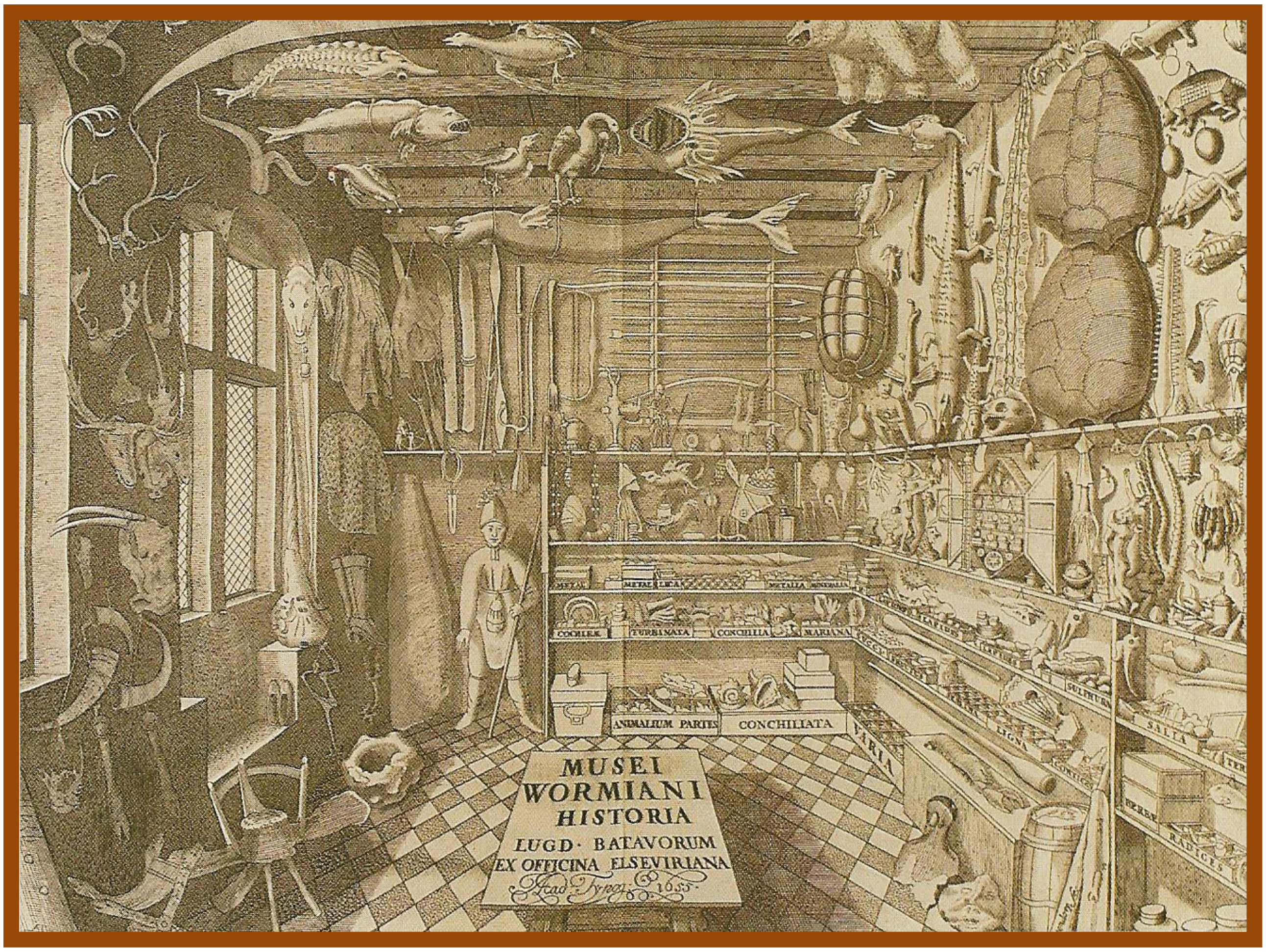
"The Secret in the Blue Blood" is dedicated to the fond memory of my father-in-law
Torben Troensegaard
who passed away on August 19, 1987, too soon to hold the book in his hands.



The collections of the famous Danish scientist Ole Worm (1588-1654) also included a horseshoe crab which appears on the extreme right on this engraving. This specimen of *Cancer moluccanus (Tachypleus gigas)* was caught alive on 4 August 1653 in Øresund, off Elsinore north of Copenhagen.

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Millions of human beings have suffered and died from diseases caused by bacterial toxins, particularly the very aggressive endotoxins, although a powerful diagnostic weapon has existed for millions of years.

From the dawn of life the blue-blooded horseshoe crabs have kept this immunological principle as a well hidden secret in the depths of the oceans, and it was not until 1956 that the American doctors Frederik B. Bang and Jack Levin triggered what would become a worldwide scientific avalanche by reporting on a peculiar property of the blue blood.

A mating couple of the Chinese horseshoe crab species, *Tachypleus tridentatus.*

Science and patience are sisters but in the mid-1970s the international scientific community finally realized that the long awaited specific diagnostic weapon against the dangerous endotoxins had indeed been discovered, and could be extracted from the blood cells of the aristocratic horseshoe crabs. The diagnostic principle was called the "Limulus Test" - and a revolution in biomedical research broke out. In 1975 the Danish author and scientist Tom Mikkelsen from the department of infectious diseases, Rigshospitalet, Copenhagen, joined the research efforts as one of the European pioneers.

In his book Tom Mikkelsen tells the fascinating story about the evolution of the horseshoe crabs through 500 million years. He takes the readers with him on his 18 thrilling underwater expeditions not only searching for hitherto undiscovered niches of horseshoe crabs, but also filming and studying the known four species of the giant horseshoe crabs in the Gulf of Mexico, on the east coast of the United States of America, off the west coast of Africa, in the Red Sea and along the wild and beautiful coastlines of India, Malaysia, Borneo, Indonesia, the Philippines, Japan, and the South China Sea.

He invites the readers to participate in his eight long and eventful visits to the People's Republic of China from 1984 to 1987, and guides the readers through the research laboratories where scientists are striving to decipher the final details of the 500 million years old immunological code in order to create test methods and principles which will make the "Limulus Test" available to even more widespread use as a guardian of health and environment in our modern society, and at the same time secure the survival of the "Living Fossils" on planet Earth for future generations to admire.

(Science Press, Beijing, about the book)

Reviews from around the World

"The Secret in the Blue Blood" is a factually convincing and truly magnificent, aesthetical monograph. Tom Mikkelsen as well as Science Press in Beijing deserve much praise for this extremely successful book".

Johan A. Wallin, M.D.*,* Research Librarian. Odense University, Denmark

Tom Mikkelsen's beautiful book is a tribute both to this amazing animal and to those scientists who have revealed its secrets.

Marlys Weary, Manager. Microbiology/Pyrogent technology.
Baxter healthcare corp., Illinois

»Tom Mikkelsen has presented science with a beautifully illustrated book which will be of great interest to a wide range of disciplines. The contribution of the Horseshoe Crab to modern medicine and to the growing understudy of the treatment of Endotoxicity cannot be over-estimated«.

Director Dr. Alan E. Preston F.R.C. (Path.) London, England

»This book is really amazing. Very well written and wonderfully provided with many gorgeous pictures. The pictures are not only of scientific value but also artistic. I congratulate Tom Mikkelsen for this wonderful book of horseshoe crabs. Everything is included: Taxonomy, paleontology, ecology, embryology, and literature. Incredible!«.

Koichiro Nakamura, Ph.D., Tokyo, Japan.

This is a fascinating, excellent account of horseshoe crabs (Limulidae) by a Danish scientist photographer/explorer. It is a combination of beautiful illustrations and a charming tale of Tom Mikkelsen's interest in Limulidae and of his world-wide search for significant populations of them.

Carl N. Shuster, Jr. - Ph.D.*,* Adjunct Professor of Biological Oceanography. Virginia Institute/School of Marine Science, The College of William and Mary Gloucester Point, Virginia 23062, U.S.A.

A fish Market - Painting by E. Snyders (1579-1657) and A. van Dyck (1599-1641), showing treasures from the sea including two horseshoe crabs.

(Kunsthistoriches Museum, Wien. Museum Number 3831)

More reviews….

»Grateful compliments to Tom Mikkelsen for his single-handed research and lifelong dedication to the study of horseshoe crabs, which has culminated in the publication of this invaluable books".

Dr. Arun Parulekar, Head, Biological Oceanography Division
National Institute of Oceanography, Dona Paula, Goa, India

With his highly entertaining and beautifully illustrated book THE SECRET IN THE BLUE BLOOD Tom Mikkelsen has made an extremely important contribution not only to the international scientific literature but also to the very friendly relations between Denmark and The People's Republic of China.

Tang Shengxi, Professor, Guangxi Medical College Nanning

The many photographs of the various genera of horseshoe crabs, the reproductions of old illustrations of this animal, and maps contribute to the value and beauty of this volume.

…… the author has provided a very readable and accessible example of the application of research in marine biology to the diagnosis of important clinical problems in humans, produced by bacterial endotoxins. Tom Mikkelsen has produced a beautiful volume that is both scientifically informative and artistically pleasing.

Jack Levin, M.D., Professor of Medicine, Professor of Laboratory Medicine
University of California San Francisco. U.S.A.

The drive, cosmopolitan nature and scientific background of the author have been the basis of this impressive work where the evolutionary history and relevant significance within human and veterinary medicine of the horseshoe crab are discussed in detail.

It is not always the case that both scientist and author are successfully combined as one and the same person. It is definitely the case here.

With his captivating talent as an author, adjusted according to scientific criteria, *Tom Mikkelsen* has, with this book, combined the past and present: zoology and medicine, history and geography.

Kaj Krongaard Kristensen, The Danish Veterinary Journal, 1988, 71, 22 15/11

A book about the world's only living fossil – the horseshoe crab – has been the cause of a justifiable sensation throughout the world, and at the same time has brought Danish scientific work into focus.

The book – *The Secret in the Blue Blood* – is written by a Dane and published by the Chinese Academy of Sciences in Beijing.

Research scientist and author, Tom Mikkelsen from Copenhagen, is the first Dane ever to have a book published in China. Others before him – among others, H.C. Andersen – have 'only' been translated for publication in the old dynasty of the east. Tom Mikkelsen has achieved this unique position for his endeavours within scientific research of the horseshoe crab and its secrets.

Birthe Lauritsen (Søndagsavisen, Denmark, October 18, 1988)

Acknowledgements

There are tens of thousands of medical professors and hundreds of millions of wives in this world. It has been my exceptional luck to meet the most outstanding representative of both categories. If I pledged that this book would never have been born without the indefatigable and forbearing participation of *Nursing Teacher, Mrs. Kirsten Mikkelsen* and *Professor, Dr. Med. Viggo Faber,* they would both be too modest to agree - and too honest to deny...

Tom Mikkelsen

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PROLOGUE

SETTING THE STAGE:

An evolutionary painting

Our precise knowledge of the birth of the Universe and of the labour is - and will presumably remain - extremely deficient.

If we go back 5 billion years we shall probably find an enormous cloud in space consisting of fragments of exploded stars, cold gases and particles of dust which were being gradually flattened out by rotation and slowly, our future sun was created in the middle of this rotating cloud. Just as the sun formed in the centre, other large fragments in the cloud at different distances from the sun became centres around which other planets of our solar system were created. The large planets are far from the centre: Jupiter, Saturn, Uranus and Neptune, and the small ones are close to it: Mercury, Venus, the Earth and Mars. We haven't forgotten Pluto which, in spite of its small size, is at the extreme edge of our solar system and takes 250 years to travel around the sun once. The planet Earth was created about 4.6 billion years ago. Its slight force of gravity and its strong heat meant that like the other small planets, it lost its original atmosphere to the deep starry space soon after its birth, but gases were released from its hot centre and created a new, secondary, reducing atmosphere, consisting essentially of hydrogen, water vapour, methane, ammonia, nitrogen and hydrogen sulphide. It contained no free molecular oxygen.

The loss of its original atmosphere left the Earth a solid planet. But the influences of pressure and energy released from radioactive processes in the crust of the Earth freed enormous amounts of heat. The Earth was, after all, too large to give off all this heat to its surrounding space, and therefore the centre of the Earth melted and was stratified, leaving the heaviest elements (iron and nickel) at the core. A mantle formed on the outside of the centre and on top of that, like the rind of an orange, the crust was created. Today, this is up to 40 or 50 kilometres thick below the continents, but below the oceans it is only about 5 kilometres in places.

The first 1 billion years of the history of the Earth are essentially unchronicled, so we must try to reconstruct events on the basis of our knowledge of later occurrences. We can assume that the temperature of the Earth eventually fell to a level which made the steam, released from the centre of the Earth through volcanic activities, condense gradually into water instead of disappearing immediately into space.

Slowly, once and for all, the ocean Panthalassa was created, of which the well-known Tethys Sea spread over areas including the present site of the Mediterranean.

In this primeval ocean we must imagine that a series of organic compounds were created through the influence of ultraviolet rays from the sun, through electric charges such as lightning, through the radioactive decomposition of the crust of the Earth, and through the heat from volcanic activities. Many theories have been advanced as to the nature of the molecules which must have been synthesized in this primitive atmosphere and in the ocean, as forerunners (progenitors) of actual life as we define it and theories are still being advanced. The list must, at least, include aminoacids for the construction of proteins, and sugars, phosphates, and organic bases for the construction of nucleic acids, and lipids for the creation of membranes - to name but some indispensable building stones.

The ocean gradually changed into a soup of organic compounds, and at some point the monomerous building stones combined into biologically active polymers. The protobionts learnt to produce copies of themselves, the organism became alive as we define it, and these particularly stable systems of reaction are the first notes in the symphony of biological evolution.

While order was being created in the orchestra pit, the stage floor was still unstable. Since creation, the crust of the Earth had been in constant motion on top of its semi-fluid mantle. Hot currents from the burning hot core of the planet took streams of nucleic matter out through the mantle. The nucleic matter poured to the sides and then made its way towards the core again. Ocean floors were lifted up and folded to enormous mountains, which were in turn eroded to plains that allowed the patient waves of Panthalassa to gain the upper hand again. The first one billion years were a gigantic geological drama of ceaseless, violent changes.

Towards the end of the long pre-Cambrian era, about 700 million years ago, and later when the large coherent continent of Pangaea appeared through the stage floor we got the first opportunities to follow the geological changes which have culminated, so far, in our present map of the world.

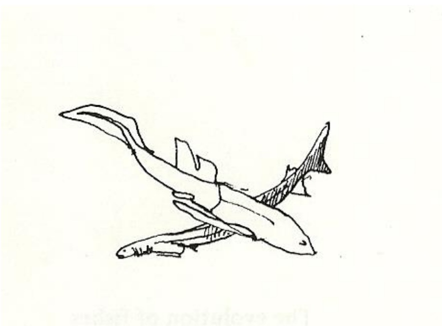
The childhood of the Earth will be forever veiled by later events, but we may look to Mars or the Moon to get an impression of the arid Earth of that time, full of deep furrows, scars, and craters. A veritable scull when we think of the fertile features of Mother Earth today.

It has been suggested that both Mars and the Moon were covered by oceans in very early times, but that the water molecules escaped the very slight forces of gravity on the planets. These possible attempts at establishing Earth-like conditions failed, at any rate, and as far as we know the Earth is the only green oasis in our solar system today, the only one of the nine planets to have oceans.

These oceans have been Life's laboratory and, seeing that the experiments were successful, Life's cradle.

When the earth was just over 1 billion years old, it was still unsettled and unstable, completely covered by a reducing atmosphere, partially covered by oceans, in whose depths the first living organisms were crawling about.

It has been commonly accepted, since the middle of this century, that the most appropriate and correct definition of living organisms will not separate plants from animals but, rather, organisms without a cell nucleus, the Prokaryotes, from organisms with a real cell nucleus, the Eukaryotes. This well-established view, then, defines only two groups of organisms as belonging to the "primitive" prokaryotes: bacteria and blue-green algae, whereas the eukaryotes comprise all green plants and animals, as well as fungi, hypomycetes, blastomycetes, and the so-called protists: flagellates, cilicates, amoebas, and a few other eukariot single-cell creatures.



The first living organisms were prokaryotes, and the oldest traces known to us today of these Clostridium-like, oxygen-shunning bacteria are fossils, about 3.4 billion years old, found in the Onverwacht deposits in South Africa. To these, the earliest prokaryotes, oxygen would have been a poisonous and corrosive gas against which they had no protection. And if, at this point in evolution, there had been multicellular animals wishing to live on land (there were none), the ultraviolet rays from the sun would, in any case, have been lethal.

As the millions of years passed, the atmosphere of the Earth changed gradually and became rich in oxygen, primarily because of the photosynthesis of the blue-green algae. It split water molecules and released oxygen into the atmosphere, initially as a waste product, because the blue-green algae only needed the hydrogen from the molecules.
These new activities gradually built up a protective layer of ozone (0_3) in the upper atmosphere. The layer kept back an ever increasing proportion of the ultraviolet rays from the sun. It made it possible for the multicellular organisms gradually to invade all niches on the Earth, but we shall stay in the sea, which had become so full of varied, prokaryote life that new inhabitants were on their way.

And at least 1.4 billion years ago, it happened: the singlecell, eukaryote organisms appeared-fossilized green algae in slate in the McMinn formation in North Australia prove the point.
Gradually, the eukaryote algae changed the sea into such an abundant larder that the invertebrates - spineless animals - could prepare themselves to see the light of day, filtered through sea water.
Today, we know of worm holes and other excavated holes and ducts in fossils which are more than 700 million years old. Fossils of molluscs, 650 million years old, have proved identical with today's jellyfish and related forms of life. Ediacara Hills in South Australia provide the greatest variety of form and mass of fossilized, precisely dated traces of this period.
We now enter the period of comparatively safe geological chronology, and we immediately note that in the earliest period of the Palaeozoic era, the Cambrian period, almost all the spineless species of animal that we know in the sea today were present then, although in more primitive forms.

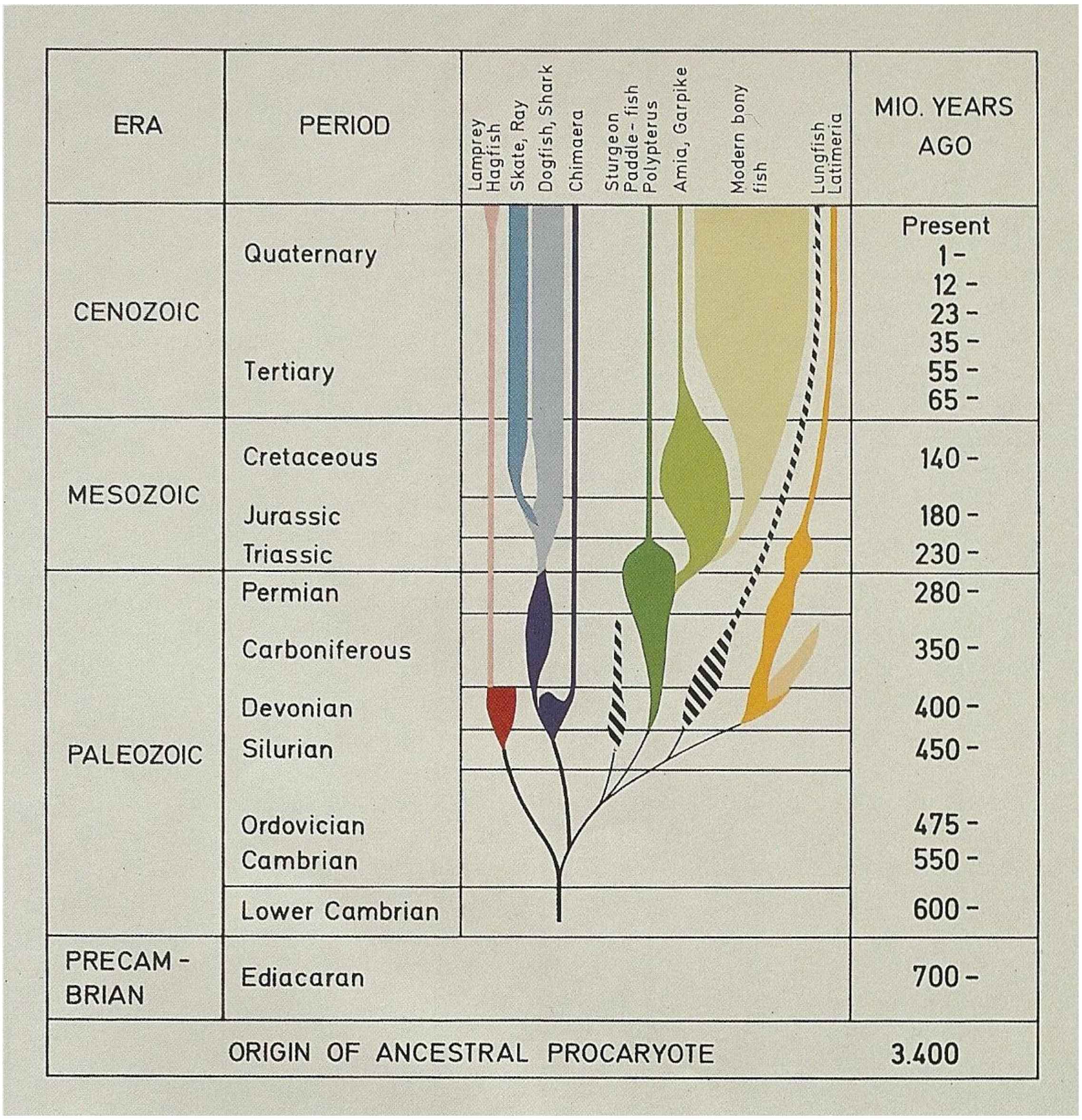
And shortly afterwards, our oldest "forefather.", the first vertebrate, swam onto the stage. Although the Cambrian period has left no known traces of vertebrates, they must have been in the early stages of their development, because we know of several fossils of vertebrates dating from the next period, the Ordovician. And such traces from the Silurian period are extremely numerous and include several versions of the first and lowest vertebrates: the ostracoderms, small jawless fish equipped with armor against the enemies surrounding them, particularly the sea scorpions - the eurypterids - voracious spineless creatures of prey up to 2.5 metres long and closely related to the horseshoe crabs, still alive today.

Both the ostracoderms and the sea scorpions died out at the end of the Palaeozoic era, but it seems beyond any doubt that the useful armor of the ostracoderms was the beginning of a later, internal skeleton of bone and that the ostracoderms were the progenitors of the cyclostomes ("round mouths"), the frightening predatory fish lamprey and hagfish, our oldest *living* vertebrates, although they exist in forms which have presumably degenerated and specialized to a considerable extent in relation to the first forms.

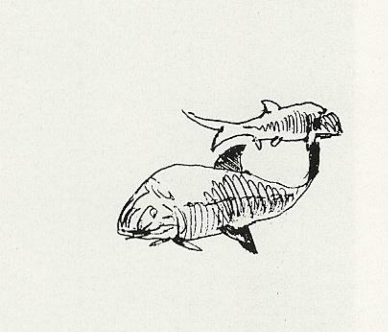
A few rungs further up the ladder of evolution there are the placoderms which appeared fully developed at the beginning of the Devonian period-the great period for fish. The placoderms were probably the dominant group of fish in the Devonian period, because it was represented all over the Earth in both salt and fresh water. Today, the placoderms are systematically divided into six orders, all of a more or less grotesque appearance, varying in size from 20-50 centimetres in the case on the acanthodians to about 9 metres for the members of the widely distributed family dinichtys.

It is probably correct to think of the placoderms as one of nature's experimenting interludes on the way to a final form of jaws and fin structures. Among the various placoderm orders, the acanthodians deserve particular attention. They appeared in the Silurian period and stayed for more than 100 million years as both fresh-water and salt-water fish before they disappeared in the Permian period. They were partially contemporary with other placoderms, but they showed no external signs of relationship with the sharks. They had good bone skeletons, strong jaws, and a body covered-not by armour and plates-but by diamond-shaped scales similar to the scales of higher species of bony fish. The order died out, perhaps because it was one of nature's aberrations, but it was not entirely aberrant, because some features of its structure, including the fins, became a great success later on in the history of evolution.

We hinted above at the difficulty involved in determining how much of the early development of the fishes took place in fresh water, perhaps lakes and rivers, and how much took place in sea or brackish water. It is even more difficult because no one really knows if the Palaeozoic ocean was salty and, if so, *how* salty.
Salty or not, the Devonian period with hot climatic zones in Europe (traces of coral reefs have been found in the Ardennes and the Rhineland) and North America, and with periods of drought in Northern Europe and Greenland, had a very rich fish fauna. They all developed jaws and were mostly fast-swimming predatory fish.



The evolution of fishes



They include cartilaginous fish (shark, ray, and a curious ratfish "chimaera") and a large group of bony fish (osteichthyes). The latter was the origin not just of the ray-finned fishes (actinopterygii) which appear today as sturgeon and pike, but also of most modern fishes (teleosts), which established themselves in small primitive, herring-like forms late in the Jurassic period, and which then became dominant from late in the Cretaceous period up to the present.

Osteichthyes also developed into the only family of rayfinned fishes which have preserved active lungs today. There are two genera of which the polypterus or bichir, as it is called, is the best-known. Polypterus is a nocturnal animal, a predatory fish 70 centimetres long, feeding on tadpoles and other small animals. It lives only in areas of Africa with yearly periods of drought, in Lake Chad for instance.

We may briefly mention the lungfish proper (dipnoi). It was quite common even in the Triassic period, and today there are five species living in South America, Africa, and Australia. But we shall conclude this fishing session for now by talking about the lobe-finned inhabitants of the Devonian ocean (crossopterygii). They represent an important development in the history of evolution.

Of all the lobe finned fishes, most attention has no doubt been given to the "impossible" blue fish, Latimeria, which was caught off the South-African coast on December 22, 1938. Everyone believed this fish to have been extinct for many millions of years, but now its home has been found off the Comoro Islands, and more than twenty such fish have been caught since.

But another lobe-finned version in the order of the rhipidistians probably had the greatest importance to continued evolution.

At this point in evolution, in the Devonian period, the sea was crowded with richly varied life, and the ozone layer kept out a sufficient amount of the ultraviolet rays from the sun, so the time had come to conquer the land—but how?

The transgression of the sea had created large and small areas of lakes, and the fishes had, of course, followed the water. Although most Paleozoic fishes are supposed to have had lungs (reduced today to an air sac which is very important to navigation), fish naturally prefer good and, if possible, deep water. Regular dry periods, and at times actual droughts, meant that the water disappeared completely sometimes, and optimum use of an atmosphere rich in oxygen presupposes qualities which the fishes never came to possess. In response to this challenge the fins of the lobe-finned rhipidistians developed with time into limbs which were well-suited to moving on land—initially in order to find water, not flee from it.

What did the large coherent continent of Pangaea have to offer anyone who might be tempted by a life on Terra Firma at the end of the Devonian period, just before the beginning of the Carboniferous period?

Let us go back one short step in evolution to say hello to the pioneers of the dry land: lichens and mosses. Lichens are closely knit families of cohabitating algae and fungi which can invade barren rocks and dry regions. They can live in surroundings which would be hostile to both kinds on their own. The lichens and other fungi and bryophytes were probably the first multicellular organisms to invade the dry land. Here, they started eroding the surfaces of rocks and prepared the way for vascular terrestrial plants. Mosses, too, are known as colonizers of barren landscapes, and although they only appear in the calendar of fossils at the end of the Carboniferous period, they may well have existed in the Cambrian or Ordovician period. Their extremely fragile structure probably explains the absence of fossils.

Today, mosses and liverworts exist in damp places all over the world, particularly in tropical areas where they grow in swamps and bogs, on wet rocks and stones, and on the ground.

A few species float on water or just below the surface, and others again exist in areas which alternate between dry and wet. Water is essential, even if it is only a thin film, for the active growth of most bacteria and algae. Land-based algae, too, thrive in damp conditions and generally go into "hibernation" in dry periods. The same is true of terrestrial fungi, particularly the mosses, which depend on water for the transfer of semen to eggs. So, these initial decomposing activities may well have lasted for a very long time, depending on the climate, but when the pioneers succeeded, eventually, in corroding the surfaces of the rocks, we reach the point when other higher types of vegetation could find a footing. We should perhaps add that the large brown algae, which were present in vast numbers in the Tethys Sea, may have created a temporary land-based relative - not surprising, when we think of the dominant presence of the brown algae along or near the coasts in the cool areas of the world today.

But back to the Devonian period when the first vascular terrestrial plants appeared: the psilophytes. Their presence is proved by the discovery of the famous fossilized Rhynia in Rhynie in Aberdeenshire. They were discovered by Kidston and Lang and confirmed a similar, but badly preserved, find made by William Dawson in Canada about 75 years earlier. In 1917, they discovered some beautifully preserved traces of the first terrestrial plants in rock formations nearly 400 million years old. These first pteridophytes, which had neither roots nor leaves, were the beginning of the incredible forests of the Devonian and, particularly, the Carboniferous periods. The individual plants were extraordinarily voluminous by today's standards. For instance, we know horsetail as a member of the Equisetum family nearly one meter high. But in those days, there were horsetail trees (calamites), which were up to 30 metres high and had stems 60 centimetres wide. The sigillaria were the forerunners of today's club moss (of the Lycopodium family). They were enormous and their bark had vertical furrows between rows of scale-shaped leaves. Lepidodendron had stems one meter wide and was given its name because of its diamond-shaped scales placed in spirals up the stem. These two last families had short rootstocks with numerous short roots. Many excellent fossils exist of this stock/root system.

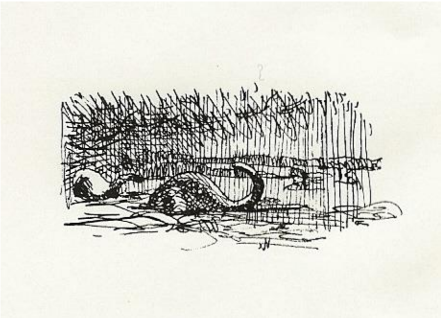
The fertile swamp forests also housed a series of species of rush with stem, leaves, and roots- and, of course, we find a mass of ferns which dominated the landscape of the Carboniferous period. The most famous example is Archaeopteris *(not* Archaeopteryx, the first feathered bird which took to its wings a little later) with leaves up to two metres long.

Among the early gymnospermous plants, the forerunners of today's conifers, we know Cordaitinea, which were more than 30 metres high, and cycas (cycadees). Among the extinct gymnospermous plants, Pteridospermae are particularly interesting, because they probably constitute the link between ferns and gymnospermous plants.

Having established the early flora of the dry land, we may now look for any possible fauna. And we do not have far to seek.

If we were to count all the insects of the world today, we would have an enormous task on our hands. We assume that today, our planet houses roughly one billion billion insects, so we are living in the era of the arthropods, more than anything else.

The original arthropods developed from the jointed worms (annelids) sometime in the pre-Cambrian era, and their body was ingenious from an evolutionary point of view with a skeleton on the outside. Modern arthropods include for example horseshoe crabs, spiders, centipedes etc. This external skeleton, shield or cuticle, consists in part of protein layers, and in part of a strong, flexible layer of chitin. Originally, it was perhaps intended for protection against enemies, but gradually it proved of essential importance to the development of life on land. This rigid, external skeleton was excellent for supporting the body of individuals moving on dry land, and it provided efficient protection against dehydration.



The first spineless animal on land may well have been the scorpion, but by far the most successful development of the arthropods resulted in the centipedes. They developed from the insects which swarmed and crawled about in the Devonian period and particularly in the forests of the Carboniferous period about 350 million years ago. Today, we know types of insect closely related to our modern cockroaches, grasshoppers, mayflies, and dragon flies. All these types had wings and could fly, which suggests that their history stretches back quite far before the time of the fossils, which can be dated with certainty as belonging to the Carboniferous period.

Some of the dragon flies had wing spans of up to half a meter from tip to tip, but we know nothing definite about the evolutionary appearance of the wings. It is certain, though, that the larder on the dry land was well-stocked, so the labyrinthodonts ("labyrinth-toothed") followed the idea of the rhipidistians and attempted, as the first amphibian-like animals, to invade the dry land. The oldest finds of the earliest labyrinthodonts, known as ichthyostegids, date back to the Devonian period and were made on the coast of East Greenland. These original quadroped ichthyostegids were obviously more closely related to the amphibia than to the lobe finned fish which were the origin of the rhipidistians, and the discovery of these very old labyrinthodonts on the Greenland coast coincides with the time when Crossopterygii were at their peak.

But the amphibian attempt at settling permanently on land was doomed to failure, because the original forms of the amphibia, just as modern amphibia (frogs, toads, salamanders, and a curious group, the apodans, which are blind and worm-like) *need* water to lay their eggs and for the larvae to develop. This problem of reproductive technique was solved by the original reptiles which appeared as cotylosaurs in the Carboniferous period, and whose descendants were to dominate most of the next era, the Mesozoic, which lasted for 140 million years.

A free life on land required, "quite simply", a terrestrial egg, this ingenious nursery with perfect room service around the clock throughout the fetal period. It was invented by the original forms of the reptiles, and this amniote egg was to become one of the most important features in the development of the vertebrates, because birds and the most primitive mammals, along with the reptiles, of course, use this invention. Complete with a protective shell, nourishment from an umbilical sac connected to the fetal alimentary canal, a large sac (amnion) full of liquid which replaces earlier watery surroundings and protects against dehydration, knocks and jolts, and finally a third sac ("allantois") which is connected to the lower part of the foetus where waste products are excreted. In a particular place, just inside the porous shell, there is a vascular membrane which is connected to the allantois. The membrane serves as a lung for the foetus: it absorbs oxygen and gives off carbon dioxide.

With their lungs, limbs, and amniote eggs, the reptiles were well-equipped for their fight to conquer the dry land of the Permo-Triassic period, about 250 million years ago. Only one other group threatened their supremacy: the sophisticated mammal-like therapsids, which appeared in the Permian period. They were carnivorous, just like their ancestors, the pelycosaurs, and had developed their motoric apparatus so that they could run quite well on four feet, like many modern mammals. The therapsids comprised 170 genera and lived all over the world as it was then, although the largest concentration of fossils (thousands) has been found in the famous Great Karroo deposits in South Africa. The therapsids lost their fight, however, against the archosaurs, enormous reptiles, and disappeared completely in the Jurassic period. *But* before they disappeared, they had provided the basis for the mammals, possibly via furry, warm-blooded cynognathus (meaning "dog's jaw") which showed many mammal-like anatomical features.

The archosaurs of the Mesozoic era, known also as "The Ruling Reptiles", have left only one living trace, the crocodile. But the archosaurs didn't develop overnight; they began quite modestly.

The best known and the earliest development of the original reptiles (cotylosaurs) were the turtles (Chelonia). We should also mention the herbivorous family of pareiasaurs that lived in Scotland, North Russia, and South Africa, and weighed up to half a ton, and Captorhinus, small, only 35 centimetres long, and carnivorous - although it was probably incapable of getting any meat other than insects. But the turtles are still here today, in swamps, on dry land, and in the sea, well-protected by thick horny plates, with limbs resembling oar blades which, along with the head, can be pulled back to safety at the least sign of trouble. The turtles have developed very discreetly; they just happen to be there.

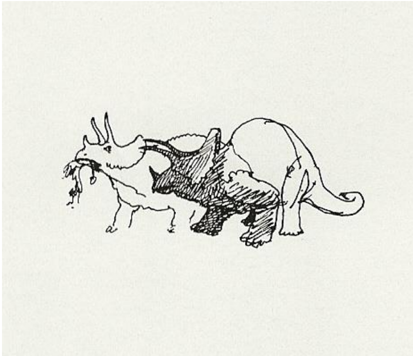
The lizards are another group of reptiles which is still represented today. They appeared in the Triassic period under the name of Lepidosauria (scaled reptiles). Today's sharply divided climatic zones mean that lizards exist in appreciable numbers only in tropical areas; they rarely survive cold spells.

The lepidosaurs also comprise the order Squamata which in turn includes saurians and snakes. The snakes probably descended from the monitor and had their prime period as late as the Tertiary period, long after most reptiles had died out. Today's boa and python represent the primitive stages of snake evolution which perhaps culminated in the poisonous types well into the Cenozoic era. Saurians, lizards, and snakes differ from turtles in that they don't really need water for living in. Only one type of modern lizard lives permanently in the sea, in areas near the coasts of the Galapagos Islands. The Hydrophidae family is the only truly ocean-going type of snake. They live in hot regions of the Indian and Pacific Oceans, they eat fish and are extremely poisonous.

The last item on this small list of our reptile predecessors on the Earth is exciting. Sphenodon or the tuatara was quite its own master, and the last living specimens exist on some small islands off the coast of New Zealand. Tuatara is the Maori word for Sphenodon (tua means "back" and tara "spine"). It used to be quite numerous in New Zealand, but hostile competitive mammals appeared and killed most of the tuatara. The New Zealand Government has intervened to preserve the last traces of a history that goes back 140 million years. The hostile mammals only reached New Zealand at the same time as man. That is why Sphenodon enjoyed this local success. Its close relations elsewhere in the world disappeared many million years ago, probably early in the Cenozoic era.

We may marvel at the profusion of archosaurs in the Permo-Triassic period. The earliest examples, the thecodonts, appeared at the beginning of the Triassic period. They scorned all attempts of the crossopterygians, the amphibians, even their own predecessors, to develop fore and hind limbs so that they could meet the hardships of life on land on all fours. The thecodonts stopped developing fore limbs and instead they developed strong hind limbs, which could carry the weight of the body in upright position. They were, literally, capable of running away from the past. One of these ungrateful creatures was Euparkeria, small and carnivorous, whose skeleton has been discovered in South Africa. Quite a few still kept all four limbs on the ground, though, and others, such as the phytosaurs, lived as amphibian.

The fore limbs simply degenerated with some of those that stood up on their hind limbs. A few types developed grasping organs, if not actual hands. Others again developed organs of flight, and then the vertebrates were ready to launch (from a tall tree) the pterosaur, the first winged reptile to conquer the air. The first flying saurian took off in the early Jurassic period and included Rhamphorynchus with strong jaws and pointed teeth, of modest dimensions (about 30 centimetres), living on a diet of fish. But the pterosaurs were not directly connected, in evolutionary terms, to the other - and greatest - event in the air of the Jurassic period. Both sensations had the same thecodont basis, but they were as different from each other as the Champs Elysée and the North Circular. We are talking, of course, of the world's first feathered, probably warm-blooded, bird: Archaeopteryx. Well-preserved skeletons and one beautiful feather of this bird have been found in Germany.



The pterosaur and Archaeopteryx, about the size of a crow, both were able to observe, at a respectful distance, all the famous dinosaurs, for example Tyrannosaurus, nearly 15 metres long and 6 metres tall, whose head was one meter long and whose awful mouth was full of 15-centimetre-long pointed and sharp teeth. But its brain was not terribly impressive, the skull was 20 centimetres long and 5 centimetres wide, about the size of the scull of a big dog.

Or Triceratops, with horns, but quite a harmless herbivore of impressive size: 6 metres long and nearly 3 metres tall. Fossils of Triceratops have been found in areas of the USA which used to be swamps. The egg thief, Ornithomimus, could be seen plundering nests with its three-fingered "hands" and also Brachiosaurus - brontosaurus - weighing 50 tons, the largest terrestrial animal the world has ever seen, or Diplodocus, whose body was about 30 metres long, the longest saurian we know of today.

So, the birds took off, but even today several types never leave the ground. The modern specimens live on the continents in the South: the ostrich in Africa, the emu and the cassowary in Australia, and the kiwi in New Zealand. The Pleistocene period, which we Holocene men have just left, also had the elephant bird on Madagascar, whose enormous eggs are discovered from time to time, complete and well-preserved in our swamps. These birds were probably able to fly once; their build is much more sophisticated than, say, Archaeopteryx. They probably stopped flying because they had no need to fly. The reptiles took to the air essentially to avoid confrontations with the terrible giant saurians, but the birds mentioned above had no carnivorous enemies and so, no real reason to fly. The African ostrich is an exception, but it lives mostly on the open savannah which allows it to see far, and it has developed into a fast runner of some size, so any modern creature of prey will think twice before it makes the attempt.

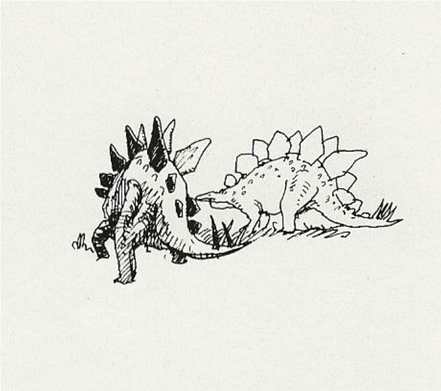
The development of a wonderful and manifold bird life from the Jurassic period to the present day is a subject which is too vast for discussion in this context, but we must mention the role which the birds played in the ecological revolution of the Cretaceous period. The revolution secured the dominance of the angiospermous flowering plants, to some extent at the expense of the gymnosperms which we met in the Devonian and Carbiniferous periods and which formed, together with the pteridophytes, the vast, but far from limitless, fuel depots.

Now, why did the angiosperms thrive all of a sudden? Was it because of the internal anatomy of the flowering plants, their – generally - wide leaves, or their contents of defensive, poisonous alkaloids? Well, there's probably more to it, because some gymnosperms also have wide leaves and their anatomy, as regards their vascular systems and transport of sap, is not necessarily very different from the angiosperms and no one knows if the alkaloids have a greater protective value against gourmets than the poisons (resins and tannins) contained in gymnosperms.

The success of the angiosperms was probably due to their appeal to the new winged insects and, particularly, the birds which could, potentially, replace wind, water, and reptiles (which never wandered very far and were far from dependable) as pollinators and seed dispersers.

Birds offered not just fast and far-ranging seed dispersal, but their behavior was stereotyped, controlled by their brain, so they returned every year and took off again for destinations which were usually quite similar in terms of climate, to their point of departure. In order to attract the attention of the birds, the angiosperms developed flowers in strong colors, fleshy fruits (often decked with hooks and prickles, just in case), and tempting nectar.

And this plan was immensely successful all over the world.



Energy is needed for flying long distances and for maintaining the regulation of the body heat. The birds eat well off the plants and often carry large quantities of seeds in their intestinal system. Their high metabolism means that most angiospermous "passengers" escape the chemical threats in the alimentary system with their capsules intact—but seeds may remain with their host bird for up to 300 hours before they are dropped, and then they are wrapped in organic material. Moreover, birds can pick up seeds directly without harming the plant, which is far better than clumsy reptiles or impatient mammals.

The fact that birds can spread seeds made it possible for the angiosperms to reach new areas where no co-adapted enemies or established diseases threatened them and, more specifically, there was no real competition from the gymnosperms. In this way, the flowering plants reached the remotest niches, small islands and areas of the continents, where they could establish themselves in peace and quiet (although it often took several bird loads). As opposed to many mammals, which were isolated to a greater or smaller extent when the continents broke up, the angiosperms could cross the oceans and join the birds in their conquest for ever larger parts of the globe, to the happiness and prosperity of both.

Of course, the angiosperms didn't entirely replace the gymnosperms, nor did they refrain from using wind and water-based pollination and seed dispersal. There are no fixed limits here. The extensive coniferous forests in Scandinavia, for example, provide ample proof of the continued relative prosperity of the gymnosperms.

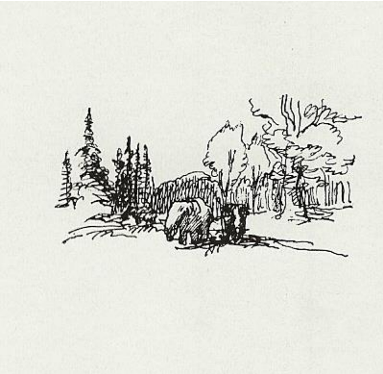
Although the mammal-like therapsids died out in the Jurassic period, this did not delay evolution, as suggested above. Their small alert successors managed to keep clear of the ravage of the monstrous reptiles, so when the archosaurs died out at the end of the Cretaceous period, these small animals managed to spread and develop a fairly high degree of organization, which meant that they were ready to make a notable impact.

These primitive mammals of the Mesozoic era were generally no larger than today's rats or mice, and may have resembled them. They probably fed on insects, worms, buds, and eggs. Their brain was still relatively undeveloped as compared to modern mammals, but it was very sophisticated compared to the reptiles. Unfortunately, the total number of fossilized primitive mammals discovered to date can be contained in a tureen.

When the archosaurs disappeared and the mammals had prepared themselves well to assume superiority, our own era began, the Cenozoic, which has lasted for a mere 65 million years. The first 64 million years constituted the Tertiary period and we are now living in the Holocene epoch of the Quarternary ("fourth") period.

The mammals dominate the dry land completely, but their number and influence in the air and the water are quite limited today. The bats are our only representatives in the air. They have descended from primitive four-legged insect-eaters, and the oldest fossilized skeletons of bats that we know today date from the Eocene epoch. They have been found in Europe and Wyoming, USA. In watery areas we are represented rather more strongly, but whales, sea cows, seals, and dolphins, although they exist in all the oceans of the world (as yet), are merely a (useful) supplement, along with the other mammals of the sea, to the marine fauna.

In the early Cenozoic era, the separation of the continents was very marked-old stretches of connecting land had disappeared and no new stretches had yet been established. All the independent continents were partly flooded from time to time, which meant that inland oceans appeared. In some places, the flooding was so violent that it cut a continent in two. This was the case in South America, where the basis of the later Amazon basin was laid in this way. Laurasia, the continent in the North, was also cut in two when the Tethys Sea and the Arctic Ocean merged.



So, when the mammals rose to fame and fortune, there were several independent continents, whereas the reptiles' Mesozoic era knew of only two large continents: Laurasia in the North, and Gondwanaland in the South. This is probably the reason why the reptiles, in their 200-million-year-long period of flowering, only produced some ten orders, whereas the mammals, in a matter of only 65 million years, produced three times that number, through both adaptive and convergent evolution. Each new isolated continent offered many, often widely different, climatic and ecological possibilities which intelligent mammals used diligently to create individual optimum forms of life.

The cerebral hemispheres of both birds and early mammals increased in terms of relative size as well as complexity, and at the beginning of the Cenozoic era the key words to success in life for individuals and particularly for groups, were: activity and intelligence-the ability to learn and to train skills was introduced in its first basic form. The Harvard laboratories have a group of pigeons that are excellent table tennis players, but the mammals do possess more skills and greater intelligence than the birds, and this is because of differences in the surface of the hemispheres, known as the cerebral cortex, or "grey matter". In a bird's brain, the grey matter is smooth and not very prominent but, particularly with Homo, it has deep folds which provide a large surface and consequently greater susceptibility to experience and training, which manifests itself, not infrequently, in intelligent behavior. The other characteristic of the mammal rulers developed gradually: a prolonged reproductive period and increased care for their new-born young.

Modern mammals are divided into 3 main groups: Prototheria (egg-laying mammals), Metatheria (marsupials that carry their living young around with them), and Eutheria (advanced mammals with placentas). The egg-laying mammals are classic examples of the geographical and ecological isolation created by the moving continents. The group numbers Platypus with its characteristic duck's bill, and the spiny anteater. They both live in Australia, the only place in the world where they still prosper, without competition in their specialized way of life, and without any notable threats from animals of prey. Prototheria lay eggs, but the young, once they hatch, drink from the mother just like other mammals.

The other monotreme, the spiny anteater, also lives primarily in Australia, but apparently it is still to be found in New Guinea and Tasmania. Platypus usually lays two eggs, but the spiny anteater lays only one, the size of a thumb nail.

A human baby born before time can complete its development in an incubator, and marsupials always give birth to their young before they can manage on their own, and therefore they have developed a permanent incubator, the marsupium.

The opossum, living in the southern regions of the USA, is the most primitive of all the surviving marsupials. A new-born opossum is the size of an eight-week-old human foetus (or of a common honeybee), but although the opossum is extremely under-developed when it is born, it does have "arms" which allow it to crawl, on its birthday (12.5 days after the fertilization of the egg with *Didelphis virginiana),* up its mother's hairy belly and into the marsupium, where it takes a firm grip of the nipple and starts drinking. If this grip relaxes the young will die, it cannot reestablish the grip.

The marsupials never succeeded in competition with more advanced placental mammals; their former and present distribution is determined by favorable geographical isolation. South America was separated from North America in the early Cenozoic era, and it probably housed quite a few advanced placental mammals, but apparently they were not carnivorous.

Therefore some interesting marsupials were allowed to develop from the opossum, some wolf-like, some cat-like - and even a sort of sabre-toothed tiger with a marsupium took the chance, At the end of the Tertiary period, however, North and South America were again connected and carnivores invaded South America. They put an end to marsupial success; only the opossum remained and it moved north.

The marsupials in Australia have had better luck. Australia appeared as an independent giant island at the end of the Cretaceous period and houses the well-known koala bear, the marmot-like vombat and, of course, the kangaroo, along with a number of other marsupials which never lay their eggs, but still give birth to babies that are not fully developed and therefore have to seek protection in the marsupium until they have become fully finished miniature copies of adults.

Before we consider the great success in recent chapters of the history of evolution - the placental mammals - we must confess that we have cheated a little as regards the reproduction of marsupials. They (the opossum) actually have a chorio-vitelline ("umbilical sac") placenta. But this type is insufficient. Many higher mammals have a chorio-vitelline placenta as a preliminary placenta, but they all develop a histologically more complex placenta from the chorio-allantoid fetal membrane.

Having confessed to this, we may now turn to Eutheria whose important characteristics are the investment of time and care in reproduction and, by necessity, a small number of offspring. Take the mackerel: it lays several hundred thousand eggs, less than a millimeter in diameter, and with enough yolk for only 3 weeks, whereas the ostrich lays one egg with a hard shelf and with 4 million times as much nourishment as the mackerel egg. When mackerels hatch, they are so small that any hungry fellow less than one centimeter long can feed on this "caviar" - the survival rate is extremely low. Ostriches guard their tough egg, and thieves are very rarely successful. Two widely different types of reproduction, but they are both sufficient to ensure the continuation of the species.

Ovoviviparity represents a further development of the ostrich's form of reproduction. The egg, once it has been fertilized, remains inside the mother until it hatches. This further development is used not just by certain vertebrates, but also by insects.

With Eutheria, the egg develops. It has no yolk prior to fertilization, but after that nourishment will be in continuous supply from the mother until her live young is born, highly developed both in terms of brain capacity, size, and general development. The ᴸegg of an elephant, initially, is no larger than that of a mackerel (and contains less yolk), but all the same it is the beginning of a 100-kilogram baby that will be born after 22 months. The foetus of our largest mammal, the blue whale, is about 8 metres long and weighs 8 tons immediately before it is born—and those 8 tons have all been amassed after the egg was fertilized: from the mother's blood circulation via placenta to her young.

The placenta of Eutheria is generally thought of as a barrier between the mother and her foetus. The mother's side of the barrier always has the endometrium (the mucous membrane that covers the uterus) as the active part of the connection, but on the foetus side it varies: the amnion sac, the umbilical sac, or the allantois sac which melts into the outer fetal membrane (chorion) to form a connection to endometrium.

This barrier of tissue may consist of different numbers of layers: 2 layers with rabbits, hamsters, chimpanzees, and human beings, 4 layers with dogs and cats, and 6 layers with cows, sheep, horses, and pigs. But with all species, the placenta works primarily to provide the foetus with nourishment, oxygen, and certain indispensable hormones from the mother's blood circulation.

At this point in evolution we can say, in all quietness, that the aggressive carnivores had enjoyed the greatest success in evolutionary history, all the way from Crossopterygii, the forefathers of terrestrial animals that feasted on (ray-finned) fish. Herbivorous offshoots had appeared from time to time, but they had always been suppressed and usually died out again quite soon. The herbivores have never been the basis for any notable higher forms of development.

Omnivorous Homo provides partial confirmation of the success of carnivorous forms of life and also represents the finest example that has yet been seen on Earth, of brain capacity and careful reproduction, two factors of monumental importance in the fight for survival.

When compared to the billions of years that we have reviewed so far, man's history of development is extremely short, but at its current point it is of decisive importance to all life on Earth.

About 50 million years ago, Africa was a freely floating island continent. A distinct flora and fauna then developed in Africa. Many species of mammal among the early colonizers started the adaptive dispersal of existing animals and plants, parallel and simultaneous with just as magnificent manifestations of life on the other island-continents: Australia and South America.

Elephants and their relatives originated in Africa, together with many lesser-known mammals. The ocean around Africa was no definitive barrier, and various species managed from time to time to cross the ocean from Europe and Asia and establish themselves on the African continent. In the Oligocene period, the precursors of pigs, buffaloes, antelopes, and lions arrived in waves to compete with - and often suppress - established forms of life.

In other words, Africa was one of the largest - if not *the* largest - centre for the development of placental mammals, and among the first to establish themselves in Africa were the primates, the monkeys.

Today, we don't know the exact origin of the primates, but an intelligent guess suggests that the primitive tree shrew may have been the founder. These insect eaters (of the order Insectivora), placental mammals from the Oriental part of the world, are illuminating examples of early mammals that survived - discreetly, by necessity - the reign of the noisy saurians.

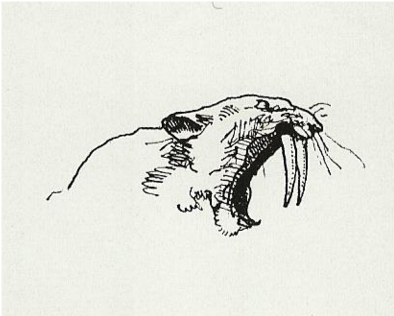
The lemurs, also known as makis, represent a very early stage in the development of the primates, and a little higher up, clearly above the lemurs in terms of development, we find Tarsius, the ghost monkey, a curious small creature that still exists. Its skull is relatively large, the eyes are big, highly developed and antrorse, its snout has been reduced to a small nose below the eyes - the beginning of a face - not very pretty, perhaps, but a face all the same.

By the time of the Eocene period, the primitive primates had established themselves in Africa. In the next 20 million years or so, a remarkable example of adaptive dispersal occurred. It produced the lemurs mentioned above and the other primitive forms: baboons and other types of Old World monkeys, anthropoid monkeys—and early man himself. At the same time, *independent* developments in South and Central America produced New World monkeys which included marmosets, spider monkeys, howler monkeys, oustiti, tamarins and others.

All mammals have good brains, but the primates - even if we exclude man - all have brains which are far bigger in relation to the size of the body than the brain of any other group of mammals.

The superiority of the primates is no doubt related to their lives in the trees which presupposed agility, muscular coordination, and well-developed motoric centres in the brain. The primates placed ever greater emphasis on vision and touch, contact by hand became much more important than the sense of smell.

In Africa, two lines of primates turned away from the life in the trees to the life on the ground, quite independently of each other: the baboons and the apes. When the apes chose to live and hunt on the savannah, they developed a completely new form of motion: running on two legs with the body fully erect.



The speed was generated solely by the hind legs below the body. In this way, the hands were "set free" which meant that eventually the apes could carry tools and weapons. 3-4 million years ago, the apes of the family Australopithecus bore themselves quite like we do, and their brain was much like that of a gorilla (the biggest known gorilla brain is 630 cubic centimetres). The apes used stones as weapons and perhaps even primitive cutting tools. The great leap ahead in man's evolution began a few million years ago, when *Australopithecus* or a similar form gave rise to the earliest "real" people of the *Homo* genus.

Between 1.5 and 0.5 million years ago, a far more sophisticated form - Homo *erectus* began to manifest itself in Africa and large parts of Europe and Asia. The brain capacity of *Homo erectus* was between that of *Australopithecus* and modern man. He knew how to use fire and advanced tools such as stone axes.

As *Australopithecus* developed it produced the idea for another ape version, *Australopithecus robustus. A. robustus* had powerful jaws and teeth which were well-suited for grinding food. So *A. robustus* was apparently a vegetarian, but *A. africanus* and *Homo* started hunting large animals on the open savannahs. Perhaps man developed into a naked ape in order to cool his body during long hunts for prey under the burning African sun? Herbivorous *A. robustus* died out just under one million years ago without ever becoming a part of the early history of Homo. Similar, short-lived offshoots appeared in the last 100 000 years of the development of modern man, *Homo sapiens.* A familiar example, is the Neanderthal man *(H. sapiens neanderthaliensis)* who lived by the Mediterranean and a little further north in Europe between 100 000 and 35 000 years ago. We also know the Solo man from Java, and the Rhodesia man from Africa.

But who was the immediate predecessor of the *Australopithecus* ape? Well, the most likely candidate today is perhaps *Ramapithecus punjabicus* known from fossils discovered in India and Kenya. But interesting traces of skeletons have also been discovered in Mount Carmel in Palestine, representing an intermediate stage between the Neanderthal man and modern man. So we still do not know the exact origin of man.

But the history of evolution led to the establishment of a complex social structure, a flexible and yet precise language, the ability to adapt to various surroundings, and profound changes in anatomy, physiology, and behavior.

For the past 700 000 years this planet has enjoyed lithospheric and hydrospheric peace (in spite of occasional harsh atmospheric/climatic periods), but socially and culturally it has most certainly seen enormous changes.

About 65 billion babies have been born since the year 6 000 BC, and they have indeed left their marks on our planet.

One of these babies, the late Professor Frederik Barry Bang of Johns Hopkins University, Baltimore, Maryland, U.S.A., called our attention to the treasure chest of scientific wisdom to be harvested from the exploration of the marine flora and fauna surrounding us.

He "reinvented" a living fossil, the horseshoe crab, and, in 1956, initiated a scientific avalanche which is still, more than 30 years later, gathering momentum on a global scale.

So let us return to the Palaeozoic sea, the cradle of life, and the natural starting point to get acquainted with the earliest relatives of our extant marine aristocrats.

PART ONE

LIVING FOSSILS: XIPHOSURA AND THEIR KIN

"Few classes offer so remarkable an instance of longevity as the Crustacea, and few orders can be compared to the Xiphosura for persistency. The Jurassic forms appear to differ little, if at all, from those of our own day, and even those of the Carboniferous epoch were at once recognized as belonging to the same family."

H. Woodward, 1867

A Genealogical Tree...

As suggested in the prologue, the 3 400-million-year-old Clostridium-like microfossils discovered in South African deposits may have been among the first manifestations of life as we define it to emerge on the planet Earth. Ever since their appearance, these procaryotes have been versatile and ubiquitous and throughout evolutionary history they have been indispensable and potentially dangerous and lethal to all subsequent higher forms of life.

They have invaded and colonized every conceivable ecological niche on *terra firma* since the emergence of Pangaea, and at the dawn of the Palaeozoic era, approximately 600 million years ago, the shallow waters of Panthalassa and the Tethys Sea were a somewhat dilute soup of procaryotes with a very significant weft of Gram-negative bacteria, just as in the coastal waters of present-day oceans where you may find upwards of one million Gram-negative bacteria per milliliter of sea-water and as much as one billion bacteria per gram of sand in near-shore areas.

The horseshoe "crabs", Xiphosura, and their kin inhabiting these ancient waters took extremely sensitive and efficient measures to protect themselves from the dangers posed by the surrounding microbes in their benthic habitat, and thereby laid the foundation for a scientific revolution which were to take place several hundred million years later.
It is quite audacious for a non taxonomist in 1988 to try to draw the genealogical tree of the horseshoe crabs, especially since eminent specialists have been disputing the subject for more than a century.

Nevertheless, I am going to try to present ancestors, cousins, other close relatives and, of course, the four acknowledged extant species of the marine aristocrats in as correct and detailed a manner as possible.

CLASSIFICATION OF HORSESHOE CRABS AND THEIR KIN

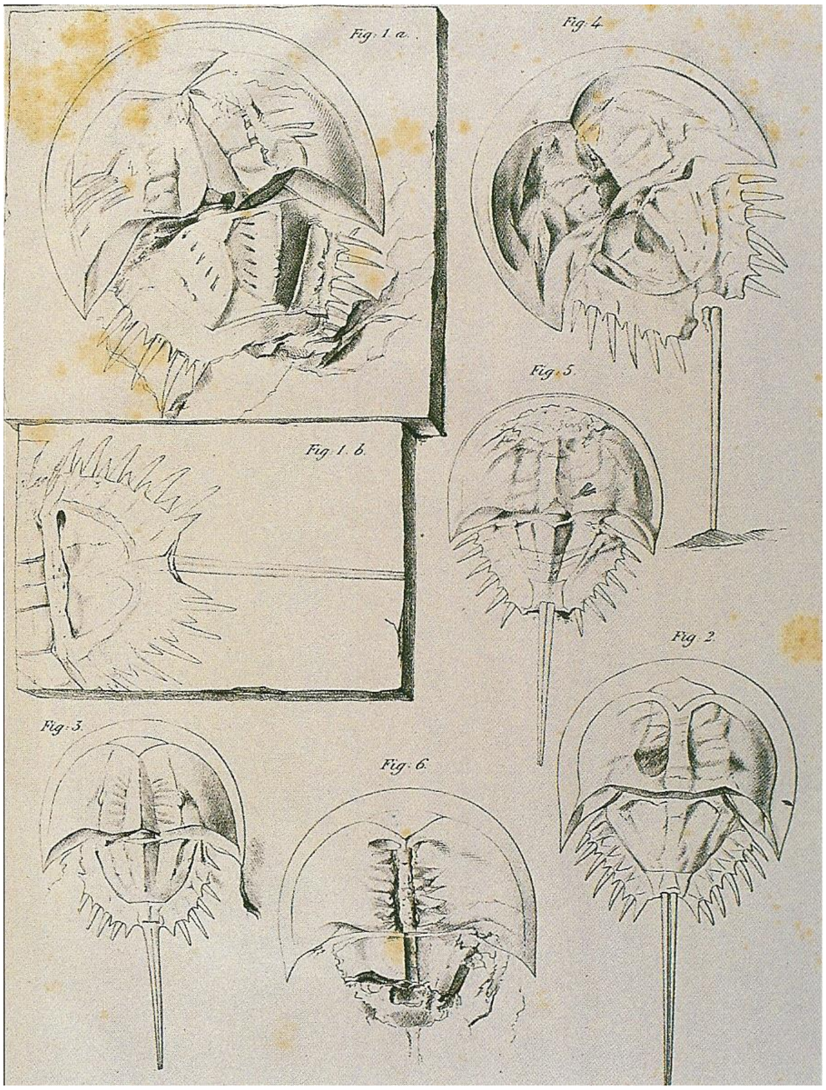
Class: Merostomata

Subclass:	Xiphosura	Subclass:	Eurypterida
Order:	Aglaspida	Superfamily:	Eurypteracea
Order:	Xiphosurida	Superfamily:	Stylonuracea
Suborder:	Synziphosurina		
Superfamily:	Limulacea		
Family:	Limulidae		

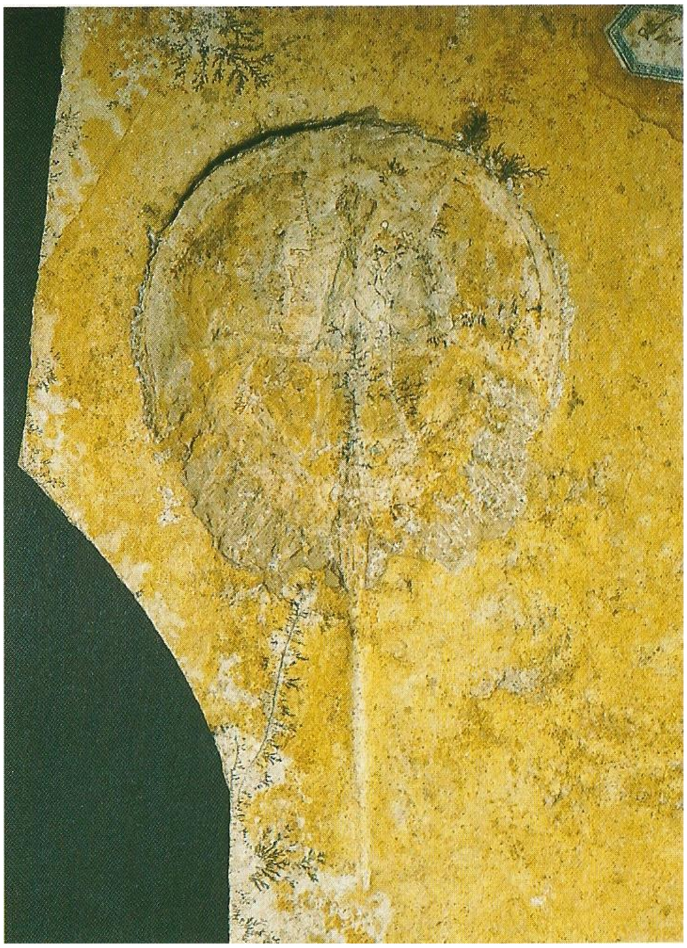
EXTANT GENERA AND SPECIES

Subfamily:	Limulinae	Subfamily:	Tachypleinae
Genus:	*Limulus*	Genus:	*Tachypleus*
Species:	*L. polyphemus*	Species:	*T. gigas*
			T. tridentatus
		Genus:	*Carcinoscorpius*
		Species:	*C. rotundicauda*

Widely dispersed millions of years ago the horseshoe crabs have left excellent fossils in various parts in the world.



A presentation of fossilized horseshoe crabs from Upper Jurassic formations. *Limulus walchi* from Solnhofen (Fig. 1 a) and from Eichstädt (Fig. 1 b). Fig. 2: *L. ornatus* (Solnhofen). Fig. 3: *L. intermedius* (Solnhofen). Fig. 4: *L. brevispina* (Eichstädt). Fig. *5: L. brevicauda* (Eichstädt). Fig. 6: *L. sulcatus* (Kelheim). (J. van der Hoeven, 1838)



A fossilized *Mesolimulus walchi* uncovered in Solnhofen, West Germany. Natural size approximately 15 cm.

Essential and obvious questions like: How many orders, infraorders, superfamilies, genera and species have roamed the shallow waters of the planet Earth during the last 500 million years? What did the ancient horseshoe crabs and their close relatives look like? How big were they? Where did they actually live? To what extent did sympatry occur? How about the life-span of the species? must all be answered in a very cautious fashion to-day.

Most of the fossilized taxa occur at single localities and stratigraphic levels, which makes estimates of stratigraphic ranges and general ecological conditions insecure. The essentially unmineralized exoskeletons of the horseshoe crabs are very poorly preserved, and especially the ventral limbs and structures hidden beneath the carapace and within the margin of prosoma and opisthosoma are difficult to recognize even when occasionally present in the matrix. But, of course, the geological record has produced some factual knowledge, and if we add the interpretations of the fossil material contributed by a number of scientists in this field and consider their hypothesis presented during the last couple of hundred years, at least tentative answers to some of the pertinent questions do appear.

One of the first fossilized horseshoe crabs to be described was *Mesolimulus walchii* (Desmarest, 1822). Hundreds of well-preserved specimens have been uncovered in the uppermost Jurassic layers of the famous lithographic limestone in Bavaria, and names like Solnhofen and Eichstädt are probably familiar.

A fatal track unearthed in Solnhofen. The track was made by the *Mesolimulus walchii* sometime during the Jurassic period. The natural size of the horseshoe crab is approximately 8 cm.

A well-preserved *Mesolimulus walchii* from Solnhofen, West Germany. Natural size approximately 10 cm.

An especially distinguishing factor connected with *M. walchi* is the remarkable "death trails" imprinted in the substrate just prior to the death of their maker. Since *M. walchi* is indeed quite similar to its extant relatives it became widely acknowledged as the fossilized horseshoe crab par excellence, although much has been learned from subsequent discoveries of a wide variety of less well-preserved specimens of other species.

From time to time, when blocks of limestone are split, well-preserved foot-prints appear, left by animals that walked or crawled across the wet sandy banks of quiet lagoons many tens of millions of years ago. Some of these prints were doubtless left by the world's first feathered bird, *Archaeopteryx,* of which no less than 6 fossilized skeletons have been found in the fine-grained limestone of Solnhofen, another proof of the importance of this area to palaeontologists. The first fossil of this first feathered flier of the world was discovered in 1861 and is now in the British Museum. One of the most recent discoveries of an *Archaeopteryx* fossil was made in 1956, just a few hundred metres from the site of the first discovery.

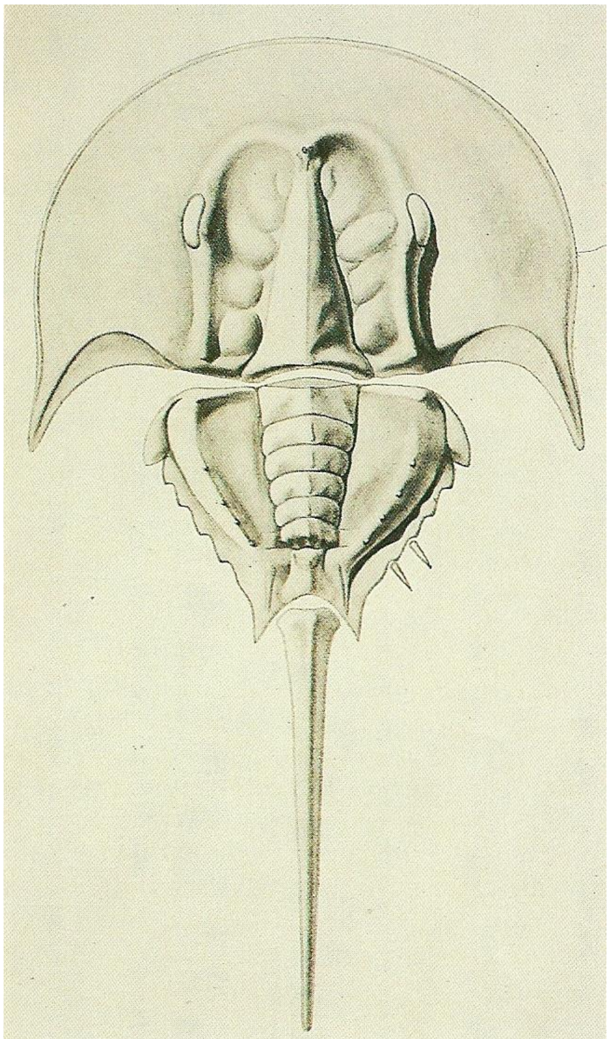
Millions of years ago, *Archaeopteryx* left its foot-prints on these muddy banks and today, seagulls, egg laying turtles, crabs and sea snails leave their prints here. And if you visit a sea shore to-day, you will find a mass, or even a mess, of overlapping prints - similarly, some of the old fossils contain layers upon layers of prints. Quite often, the prints are, of course, all different.

Most of us have probably tried - frequently in vain - to "identify prints" while searching on a beach for amber or other treasures left for us by the sea after a storm. The fossilized prints of distant ages present even greater mysteries and challenges to scientists. They have become quite good at distinguishing different types of print and at making comparisons between prints and the animals that we know were alive at the time the prints were made but, needless to say, many prints have still to be identified.

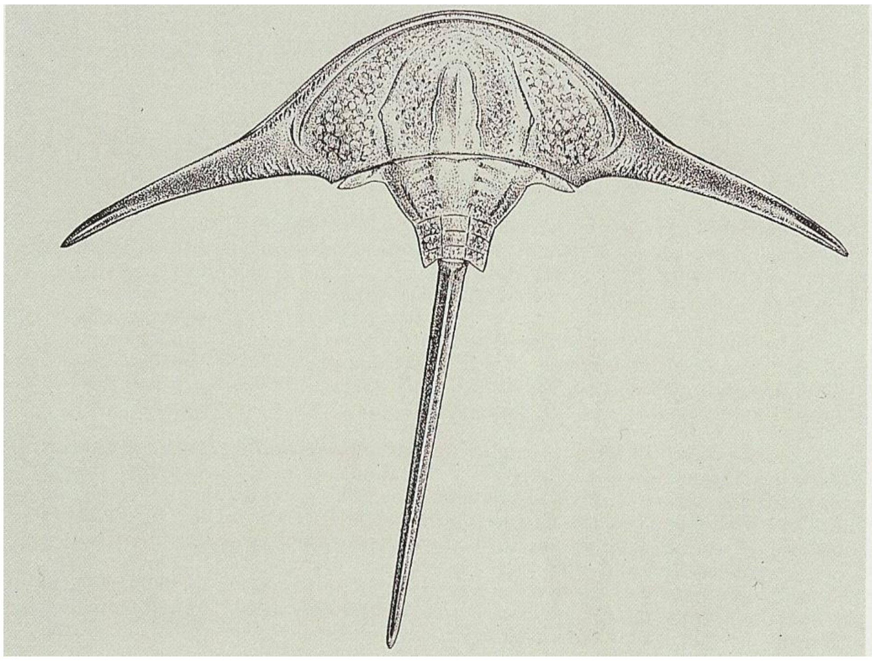
Among the Solnhofen prints, those made by birds seem particularly interesting. An imaginary name was therefore given to the originator of each individual type of print so that they could be discussed in a clear and specific way at international meetings and in scientific dissertations.

One such fictitious originator was the bird *Ornichnites caudate*; the print was believed to have been left by a bird dragging its tail. Another was named *Protornis bavaria* ("old bird of Bavaria"), a third *Hypernithes jurassica* ("the bird of the jurassic period which walked on top of the other foot-prints") and finally, "the printer of the lithographic limestone", *Koupichnium lithographicum.* Then they only needed corresponding skeletons of birds, preferably with their claws and feathers intact.

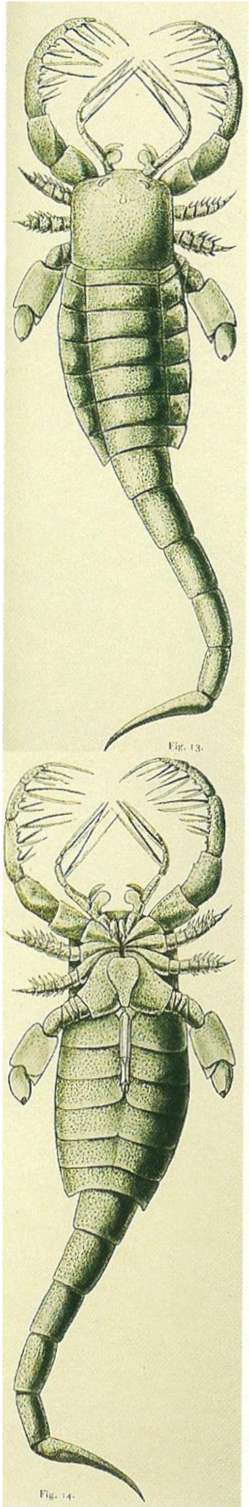
And one fine day in 1938, all these discoveries of prints culminated in one stunning revelation. Another block of fine grained limestone was split, revealing another well-preserved mass of different overlapping prints, but this time all the prints led to, and stopped at, a beautifully preserved horseshoe crab.



A restoration of *Palaeolimulus avitus* from Kansas, U. S. A.



Austrolimulus fletcheri. From the Triassic period, Australia.



Eurypterida (including the famous genera *Eurypterus* and *Pterygotus)* were close relatives of Merostomata. The Seascorpions appeared in late Ordovician and became extinct during the Permian.

One and the same fossil was identified to be *Ornichnites caudata* with its dragging tail, and the prints of *Protornis bavaria* which had in fact been left by the walking legs on a horseshoe crab. The fossil also included the prints of "the bird of the Jurassic period that walked on top of the other foot-prints", they had been left by the sixth pair of prosomal appendages, the "snowshoes". And finally, it also identified the horseshoe crab as the true "printer of the lithographic limestone", the prints had been left in the mating season by a wandering female with a male attached to her opisthosoma - a tandem of horseshoe crabs.

When the true identity of all these fictitious birds had been revealed, the geologists proceeded to investigate mysterious fossilized prints left at an even earlier point in geological time. Extremely detailed and expert work revealed 360-million-year-old prints in rock formations in Pennsylvania to be the prints of horseshoe crabs. They were originally believed to have been left by an intermediate form linking amphibians and reptiles (known as *paramphibius).* Interestingly enough, these prints were virtually indistinguishable from prints made by horseshoe crabs today.

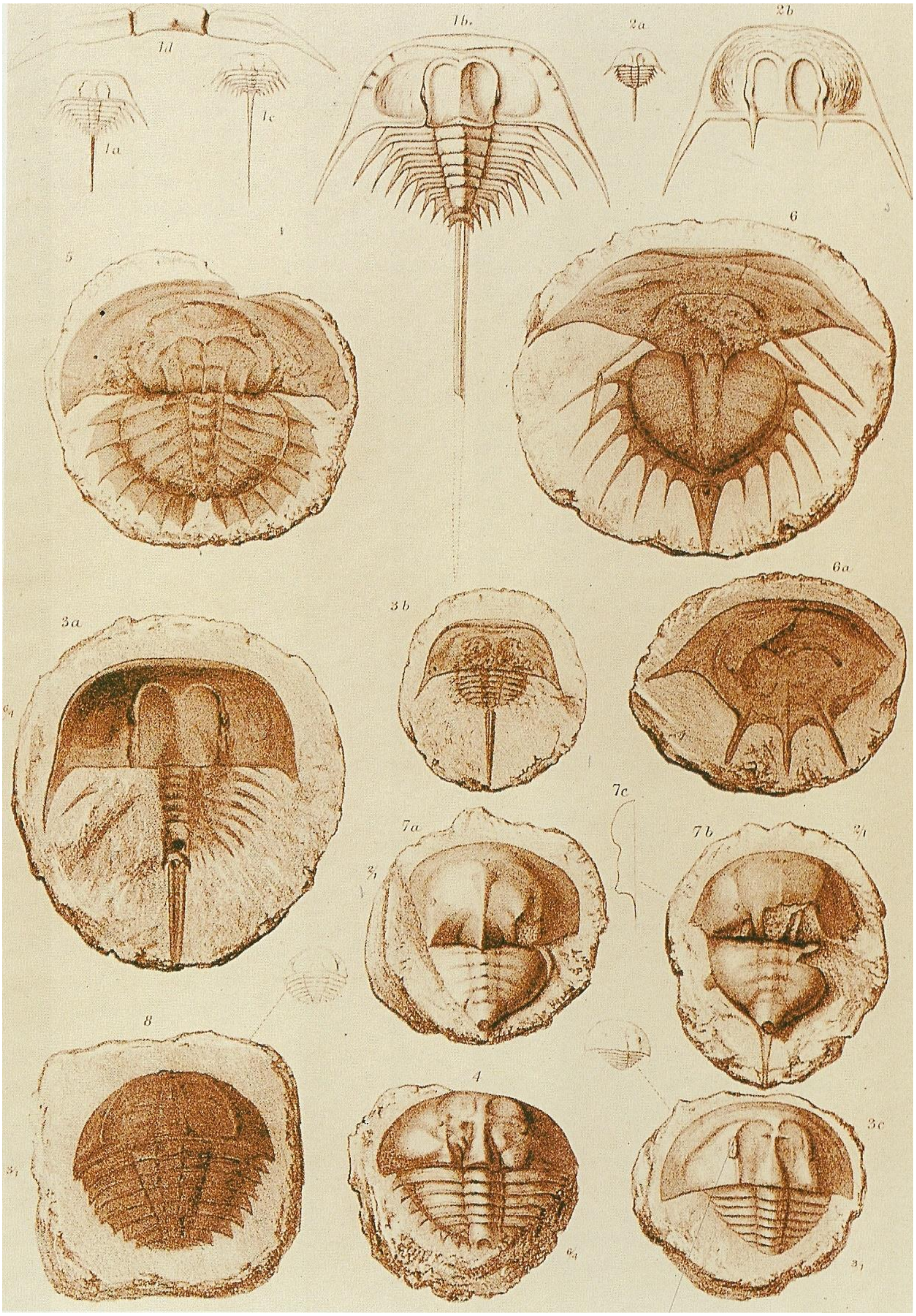
Similar work was done in New Jersey on two sets of prints from a "small hopping dinosaur" and in Greenland on the supposed foot prints of an enormous newt. All these fossilized prints matched perfectly the prints of horseshoe crabs 1988.

No wonder that the horseshoe crab has acquired the well-earned title of "The greatest track-maker in history" after hundreds of millions of years of moving about on the sea shores of the world.

A number of fossilized species of horseshoe crabs have been found in strata ranging from Triassic to Oligocene. The earliest of these *(L. vicensis)* comes from the Buntersandstein of the Vosges and the Keuper of Lorraine. *L. decheni* was found in the Oligocene brown coal of Teuchern near Merseburg. *Mesolimulus syriacus* was identified in the Cretaceous strata of Lebanon, *M. nathorsti* appeared in Jurassic layers in Sweden, while England gave us *M. woodwardi* and Germany presented us with *Psammolimulus gottingensis..* From the lower Triassic layers of France we have the above mentioned *Limulitella vicensis.* The United States have contributed *Palaeolimulus avitus* from the Permian layers in Kansas and *Limulus coffini* from the Cretaceous layers in Colorado, just as Australia introduced *Austrolimulus fletcheri* from Triassic formations.

Venturing further back in the geological records we meet such fascinating relatives as *Aglaspida,* the gigantic *Eurypterida,* and also the order *Xiphosurida* which may conveniently be subdivided into *Synziphosura* and *Limulina.* The *Eurypterida* is an order of extinct marine *Chelicerata* completely restricted to the Palaeozoic era. A number of the genera of *Eurypterida* seem to have originated in the Ordovician, but not to have survived to the Silurian. *Pterygotus* ranged from late Ordovician to early Devonian. It was a gigantic form with long chelate chelicerae and it is best known in England from the Old Red Sandstone. *Eurypterus* existed from late Ordovician to the Permian and it reached a length of three feet. It is found only occasionally in Old Red Sandstone and its classic locality is the island of Oesel, where specimens from the fine Silurian marl are perfectly preserved. *Stylonurus,* reaching a length of ten feet, ranged from the late Ordovician to the early Carboniferous. The group was at its highest point of development in the late Silurian and judging from the geological records, many of the genera of Eur*ypterida* were short-lived.

From the organisms with which they were associated, it is believed that the *Eurypterida* were originally marine forms and that they became gradually adapted to brackish and maybe even fresh water surroundings.



A manuscript drawing from 1878 depicting *Belinurus* species (Fig. 1 to 4), *Prestwichia* species (Fig. 5 to 7) from the Carboniferous period, and a specimen of *Limulus falcatus* (Fig. 8) stemming from the Upper Silurian period. All Specimens have been found in Great Britain.

They appear to have been sluggish, crawling benthic creatures, although one genus, *Dolichopterus,* included good swimmers, and *Pterygotus* was most likely insidiatorial.

The *Aglaspida* have been considered the sister group of the *Xiphosurida,but* existing evidence has recently been questioned. Rather than a well-defined sister to the *Xiphosurida,* the *Aglaspida* appear to be a series of groups, such as chasmatapids, diploaspids and others, whose relationships, however, have yet to be clarified.

The *Aglaspida* seem to have lived in shallow seas in the company of trilobites and brachiopodes between lower Carboniferous and upper Ordovician times. The general shape of the body in some genera of *Aglaspida* is similar to that of *Eurypterida* and *Synziphosura,* but although the *Aglaspida* also possess the characteristic, powerful telson, there are considerable differences between an *Aglaspid* and a horseshoe crab of to-day. *Aglaspida* are considered representatives of the most primitive kind of *Xiphosura,* and Strabops is a typical genus.

Members of the group *Synziphosura* comprise, among others, the genera *Bunodes* from Oesel and *Hemiaspis* from Scotland. All *Synziphosura* are extinct, and fossils have been found together with *Eurypterida* and the earliest chordates in the upper Silurian and lower Devonian strata. It is believed that some of these animals were able to protect themselves against predators by rolling up like a woodlouse, and in many of them no trace of eyes can be discerned - quite contrary to e.g. *Eurypterida* that possessed a median pair of simple ocelli and large, bean-shaped lateral compound eyes, just as in the extant species of horseshoe crabs.

The phylogenetic structure of the infraorder *Limulina* is of great interest, for its representatives are found from the Devonian to the present day.

A reconstruction of the ancestor - descendant sequence of the three superfamilies concerned, based on stratigraphic occurrence of the taxa, has been proposed to look this way:
Belinuracea -> Euproopacea --> Limulacea (Palaeolimulidae -> Mesolimulidae -> Limulidae).

The genus *Belinurus* had a carapace closely resembling that of the *Synziphosura, Euproops* of the upper Carboniferous bore a strong outward resemblance to *Limulus,* and in the third superfamily are placed the extinct *Palaeolimulus* from the Permian, the *Mesolimulidae* from the Triassic and Jurassic and the *Limulidae* of today.

The best available data on the interesting question of the period of existence of various species are from the Carboniferous, where several species (e.g. *B. reginae, B. koenigianus, E. rotundatus)* are recognized as having ranges of about 15-20 million years. It is unknown, however, how typical the periods of existence of these horseshoe crab species may be.

Information about geographic distribution, the general ecology of the fossilized horseshoe crabs and the incidence of sympatry up through geological history is subject to a variety of sampling problems and thus to considerable dispute.

Most fossilized horseshoe crabs have been found in brackish or/and freshwater environments. Was it because they died and were buried during their annual mating migration, away from "home"? Or was it because the possibilities of preservation are better in these environments than in traditional marine habitats? Did the ancient horseshoe crab species actually migrate to shallow (coastal?) waters during the breeding season, just as the extant species do—or is it possible that they lived in those brackish or/and freshwater habitats permanently?

The majority of morphologically plesiomorphic taxa from *Neobelinuropsis rossicus* to the extant species inhabited nearshore marine environments. Among the advanced *Belinuraceans* and *Euproopaceans* one genus, *Euproops,* was probably amphibious to some extent, while other *Euproopaceans* were restricted to the freshwater areas of the Carboniferous coal swamps, as proved by relatively complete ontogenetic series of fossils.

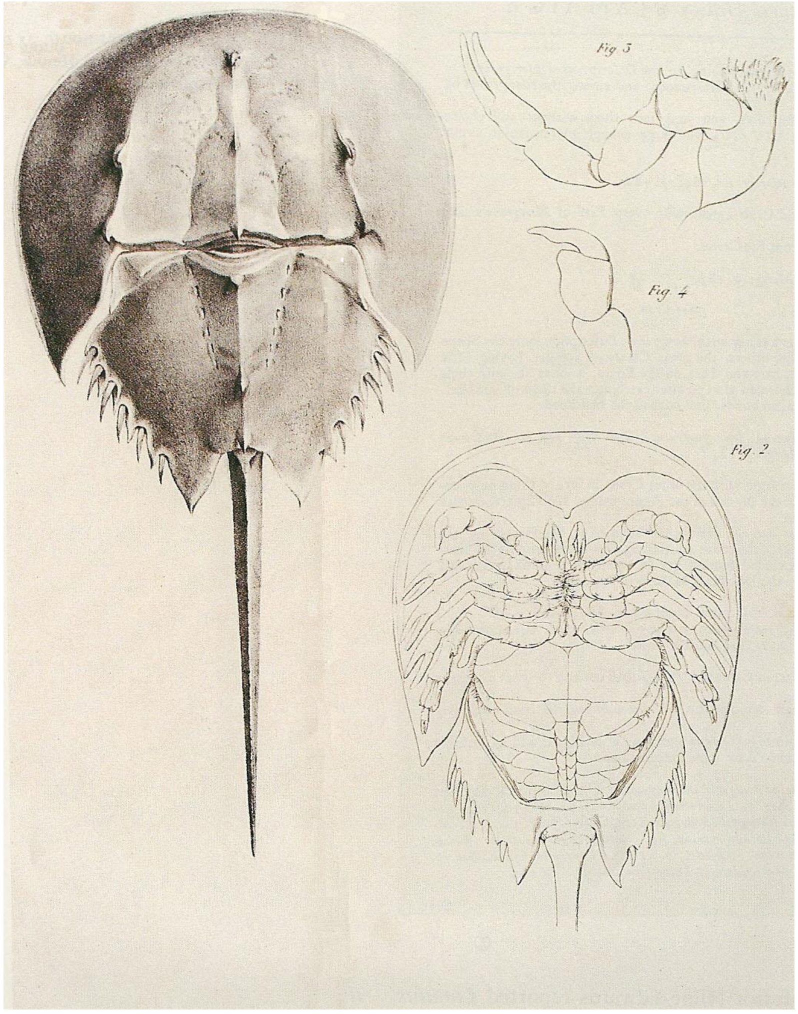

An ancient river mouth as seen by a Danish artist.

With regard to sympatry among the fossil species, a *Synziphosuran* may occur together with a *Pseudoniscine,* as is the case of *Bunodes lunula* and *Pseudoniscus aculeatus* in the upper Silurian Oesel fauna from the Baltic. Among *Limulina,* sympatry appears to be at its highest during the Carboniferous where one may find a predominantly marine species (like a *Palaeolimulid* or a *Belinurid)* together with a brackish species (maybe also a *Belinurid),* a freshwater species and one or two species occupying small freshwater streams *(Euproopids).* Probably the best example of local sympatry involved the *Euproopid* species *E. rotundatus* and *E. anthrax* in Great Britain. It has not been possible to prove sympatry between marine species, except maybe the species *Heterolimulus gadeai* and *Tarracolimulus rieki,* uncovered side by side in the Jurassic strata of Tarragona, Spain.

Geographic ranges are generally extremely difficult to assess with any degree of certainty, but it has been done successfully with the Carboniferous species *Euproops danae,* which occurs throughout much of North America from Illinois to Canada, just as *E. rotundatus* and *Belinurus reginae* are known from a number of localities in Great Britain and Europe. But, again, it is unknown whether such ranges are typical of other fossilized horseshoe crabs.

When we turn to the four extant species of horseshoe crabs (considering *T. hoeveni* as described by Pocock in 1902 to have been a somewhat abnormal specimen of *Tachypleus gigas)* it would be interesting to be able to pinpoint a common ancestor.

And at the initial split between lineages leading to the three Indo-Pacific species in one direction, and to American *Limulus polyphemus* in the other, we do find *Tachypleus decheni* and *Limulus coffini* (and, in addition, the Maestrichtian *Castrolimulus kletti,* which may also be associated with the lineage leading to the extant Indo-Pacific species). The identification of *L. coffini* gives a *minimum* of about 75 million years for the most recent common ancestor.



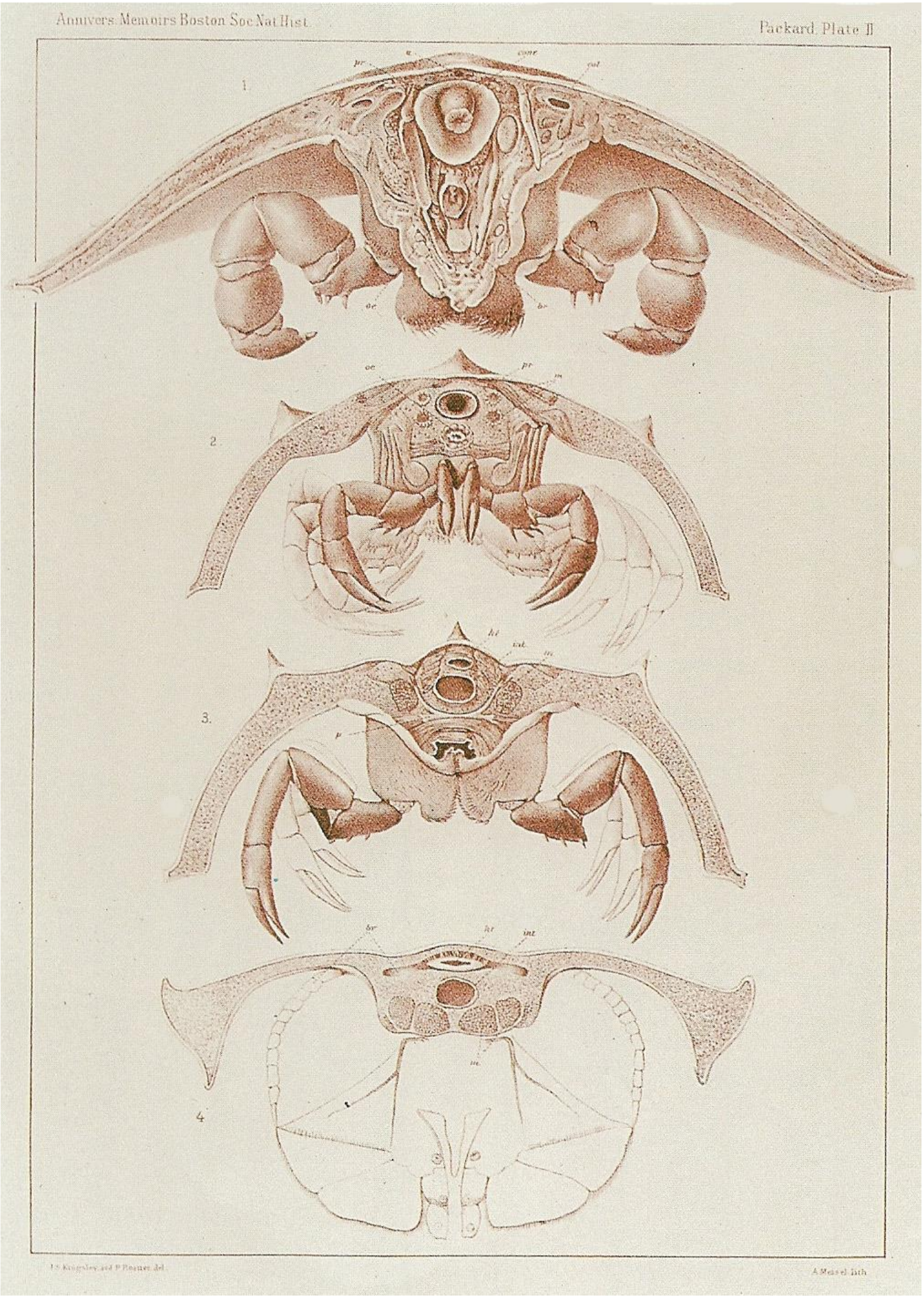
Female (Fig. 1) and male (Fig. 2) of *Limulus polyphemus.*
(J. van der Hoeven, 1838)

Living Fossils

In their delightful book *The Crab That Crawled Out of the Past* Lorus and Margery Milne state that ".... the west coast of America has no horseshoe crabs. Nor have any shores of South America, Africa, Europe or Australia". This statement has generally been accepted by all involved researchers and, although this author will dare to challenge this and other statements in later chapters, there are indeed only two areas in the world where horseshoe crabs may be encountered with certainty, and there are only four acknowledged species in three genera left.
Thanks to prolific American writers and scientists, notably Margery and Lorus Milne, Carl N. Shuster, Jr. and Anne and Jack Rudloe, the most detailed data on geographical distribution, general ecology, breeding behavior and population sizes are available for the American horseshoe crab.

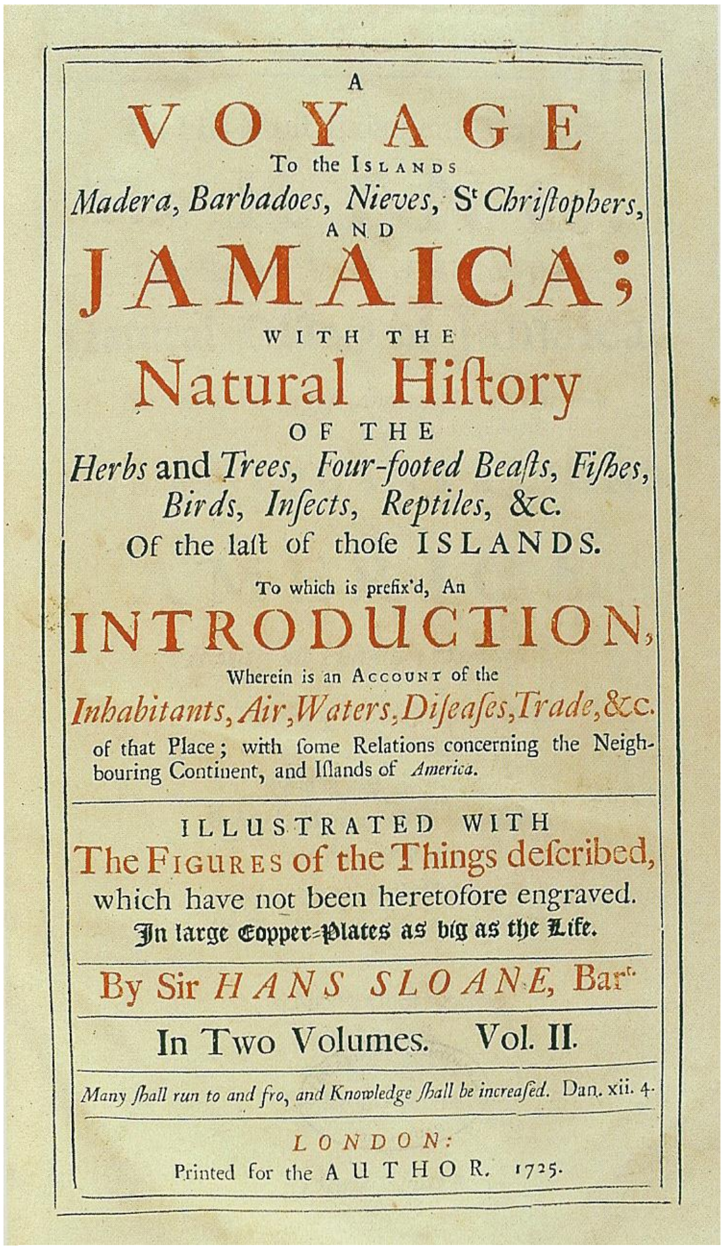
Limulus polyphemus

Limulus polyphemus is a benthic creature found along the east coast of North America from Maine to the Florida Keys and around the rim of the Gulf of Mexico to as far south as the Yucatan Peninsula. It has often been stated (also by this author) that *L. polyphemus* lives as far north as Nova Scotia, but apparently this has been based on very slight "evidence":



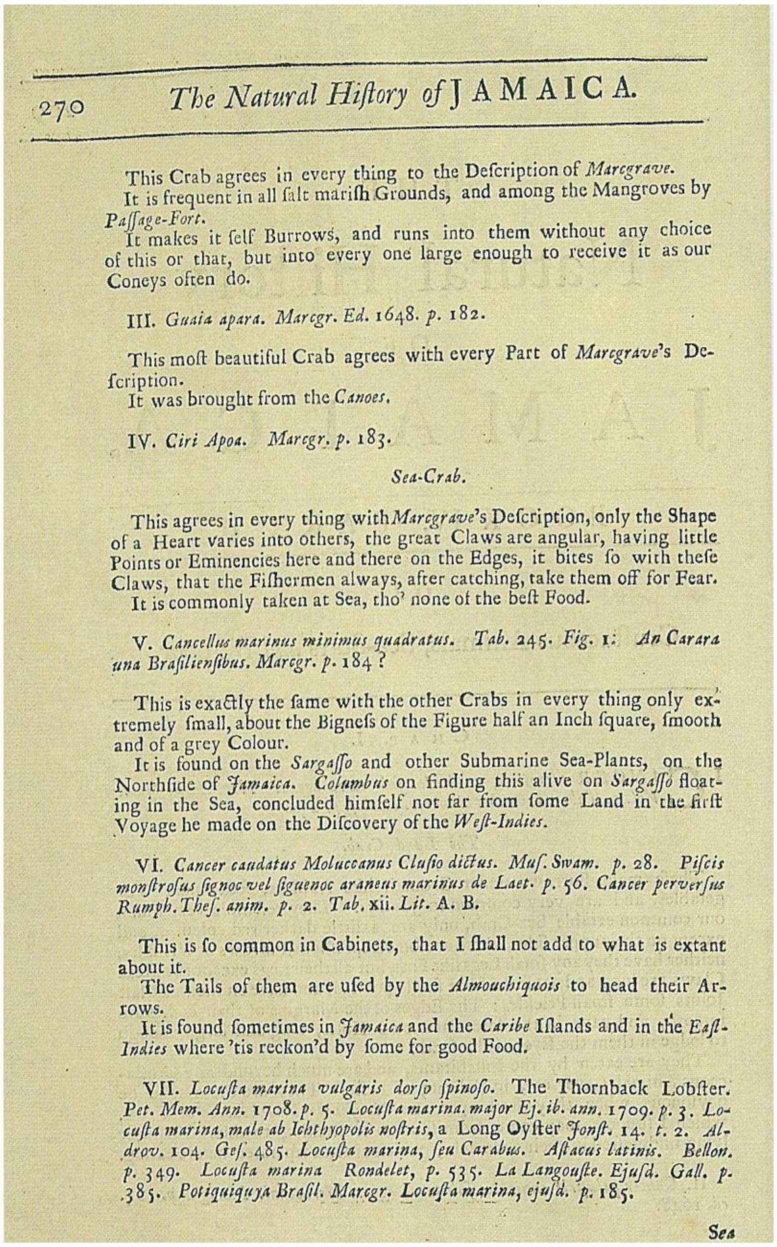
"Sections through adult king crab" *(L. polyphemus* male).

(A. S. Packard, Jr. 1872)



A
VOYAGE
To the ISLANDS
Madera, Barbadoes, Nieves, S^t *Chriſtophers,*
AND
JAMAICA;
WITH THE
Natural Hiſtory
OF THE
Herbs and *Trees, Four-footed Beaſts, Fiſhes, Birds, Inſects, Reptiles,* &c.
Of the laſt of thoſe ISLANDS.
To which is prefix'd, An
INTRODUCTION,
Wherein is an ACCOUNT of the
Inhabitants, Air, Waters, Diſeaſes, Trade, &c.
of that Place; with ſome Relations concerning the Neighbouring Continent, and Iſlands of *America.*
ILLUSTRATED WITH
The FIGURES of the Things deſcribed,
which have not been heretofore engraved.
In large Copper-Plates as big as the Life.
By Sir *HANS SLOANE*, Bar^t.
In Two Volumes. Vol. II.
Many ſhall run to and fro, and Knowledge ſhall be increaſed. Dan. xii. 4.
LONDON:
Printed for the AUTHOR. 1725.

A very long title….



270 The Natural Hiſtory of JAMAICA.

This Crab agrees in every thing to the Deſcription of *Marcgrave.*
It is frequent in all ſalt mariſh Grounds, and among the Mangroves by *Paſſage-Fort.*
It makes it ſelf Burrows, and runs into them without any choice of this or that, but into every one large enough to receive it as our Coneys often do.

III. *Guaia apara. Marcgr. Ed.* 1648. *p.* 182.

This moſt beautiful Crab agrees with every Part of *Marcgrave*'s Deſcription.
It was brought from the *Canoes.*

IV. *Ciri Apoa. Marcgr. p.* 183.

Sea-Crab.

This agrees in every thing with *Marcgrave*'s Deſcription, only the Shape of a Heart varies into others, the great Claws are angular, having little Points or Eminencies here and there on the Edges, it bites ſo with theſe Claws, that the Fiſhermen always, after catching, take them off for Fear.
It is commonly taken at Sea, tho' none of the beſt Food.

V. *Cancellus marinus minimus quadratus. Tab.* 245. *Fig.* 1. *An Carara una Braſilienſibus. Marcgr. p.* 184?

This is exactly the ſame with the other Crabs in every thing only extremely ſmall, about the Bigneſs of the Figure half an Inch ſquare, ſmooth and of a grey Colour.
It is found on the *Sargaſſo* and other Submarine Sea-Plants, on the Northſide of *Jamaica. Columbus* on finding this alive on *Sargaſſo* floating in the Sea, concluded himſelf not far from ſome Land in the firſt Voyage he made on the Diſcovery of the *Weſt-Indies.*

VI. *Cancer caudatus Moluccanus Cluſio dictus. Muſ. Swam. p.* 28. *Piſcis monſtroſus ſignoc vel ſiguenoc araneus marinus de Laet. p.* 56. *Cancer perverſus Rumph. Theſ. anim. p.* 2. *Tab.* xii. *Lit.* A. B.

This is ſo common in Cabinets, that I ſhall not add to what is extant about it.
The Tails of them are uſed by the *Almouchiquois* to head their Arrows.
It is found ſometimes in *Jamaica* and the *Caribe* Iſlands and in the *Eaſt-Indies* where 'tis reckon'd by ſome for good Food.

VII. *Locuſta marina vulgaris dorſo ſpinoſo.* The Thornback Lobſter. *Pet. Mem. Ann.* 1708. *p.* 5. *Locuſta marina. major Ej. ib. ann.* 1709. *p.* 3. *Locuſta marina, male ab Ichthyopolis noſtris,* a Long Oyſter *Jonſt.* 14. *t.* 2. *Aldrov.* 104. *Geſ.* 485. *Locuſta marina, ſeu Carabus. Aſtacus latinis. Bellon. p.* 349. *Locuſta marina Rondelet, p.* 535. *La Langouſte. Ejuſd. Gall. p.* 385. *Potiquiquya Braſil. Marcgr. Locuſta marina, ejuſd. p.* 185.

Sea

….. and some interesting information about Limulus polyphemus from 1725

A dead specimen found near Digby Neck in 1955, and a living specimen caught off La Have Islands in 1956. In 1880 Professor Milne-Edwards reported *Limulus* to be abundant in Laguna de Terminos at the southern extremity of the Gulf of Mexico, and, in 1891, Ives suggested that it may be present along the South American coast between Islas de la Bahia and the eastern coast of Yucatan.

Old books on the fauna of the West Indies describe the Horseshoe crabs on the coast of Jamaica.

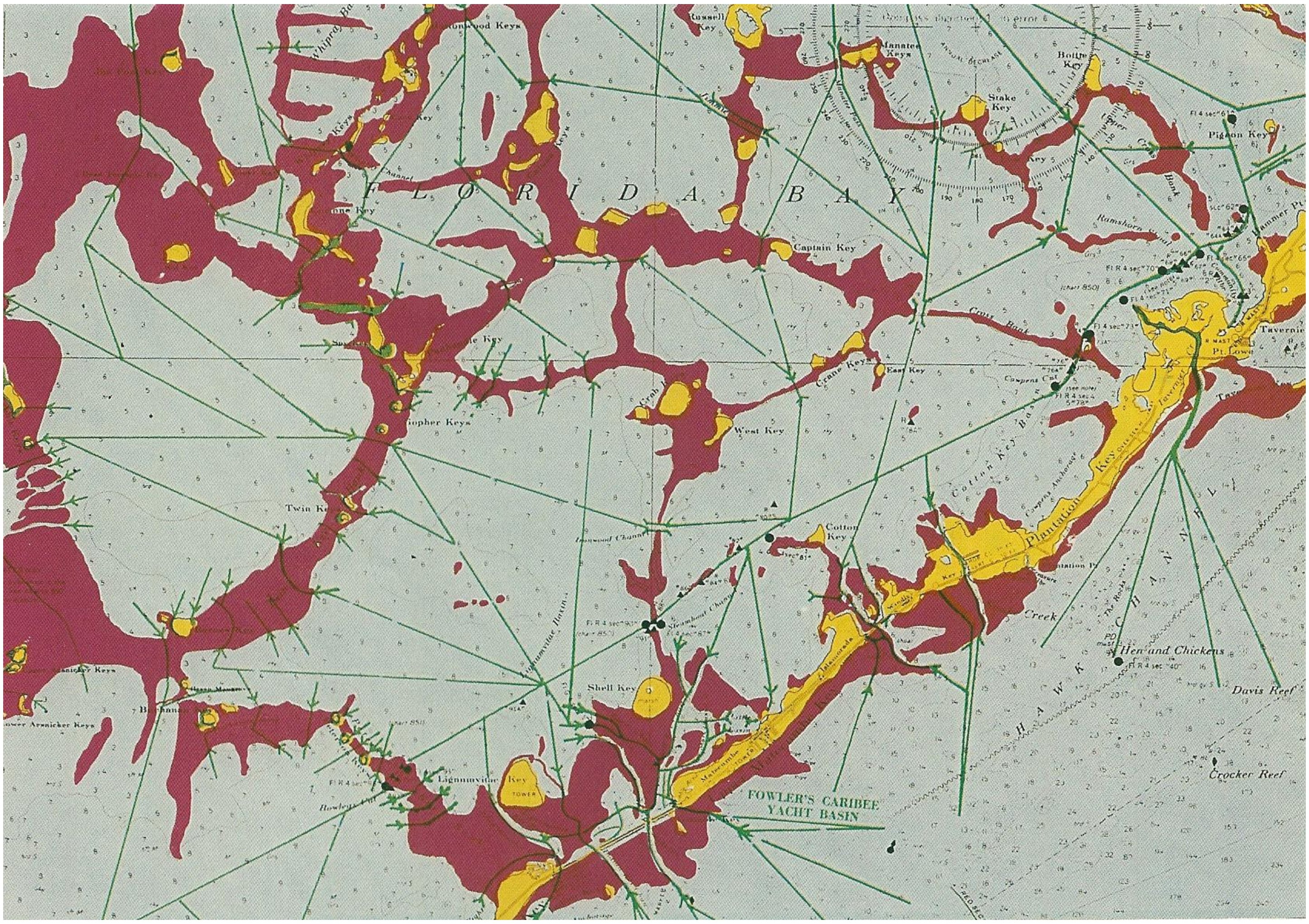
The largest limuli found on the coasts of Yucatan and Campeche may reach 310 mm in prosomal width and are thus considerably smaller than their relatives living in the middle of the geographical range.

But stating that *L. polyphemus* ranges from Maine to Yucatan on the Atlantic and Gulf coasts of North America may not be sufficient to actually find a horseshoe crab. The species is broken up into a series of relatively discrete populations concentrated more often than not around estuarine systems and coastal lagoons providing the sheltered beaches and intertidal flats apparently preferred for reproduction and juvenile development. While a large hiatus exists along much of the Gulf coast, *Limulus* populations are found at each of the major estuaries along the eastern coast of the United States.

Dr. Carl Shuster has provided a detailed map of the distribution of *Limulus* on the continental shelf of the Atlantic coast of the United States from north to south, also identifying the area of the associated estuarine habitats. The map comprises Plum Island Sound, Cape Cod Bay, Nantucket Sound, Vineyard Sound, Delaware Bay (with probably the largest population of horseshoe crabs on the Atlantic coast, amounting to hundreds of thousands), Chesapeake Bay, Oregon Inlet in North Carolina, and along the South Carolina coast the list contains localities such as Bull Bay, St. Helena Sound and Port Royal Sound. Further south, in Florida, you may find horseshoe crabs in Lake Worth, Boca Raton and Biscayne Bay.

During three stays on the Florida Keys in 1981,1983 and 1985, each stay lasting 2-3 months, we had ample opportunity to study the horseshoe crabs along the who--le beautiful necklace of island pearls on both the Atlantic and Gulf coasts. Our favorite locations are Cotton Key Basin (off Plantation Key), Cape Sable (on the southern tip of the Everglades), Lower Matecumbe Key (Channel Two) where we have found females of *L. polyphemus* exceeding two feet in length (including the telson), which is quite normal in certain locations despite their deep southern position. Lignumvitae Key, Bahia Honda, Long Key and Key West are other good locations, and, finally, you may go by boat to the Marquesas Keys off Key West in the Gulf of Mexico. To camp on the Marquesas Keys during the breeding season is a wonderful experience for a student of horseshoe crabs.

The Marquesas Keys are completely deserted except for myriads of aggressively hungry insects led by mosquitoes and thousands of horseshoe crabs in crystal clear water or on the sandy beaches. The Marquesas Keys encircle a huge intertidal mudflat where you may easily find juvenile horseshoe crabs of virtually all sizes. The waters surrounding the Marquesas Keyes are also breeding ground for sharks, so scuba divers and snorkelers may have additional adventures if they are a little cautious.



A chart of part of Florida Keys. If you are able to navigate north through the shallow and dangerous channels and avoid the extensive mudflats, you will eventually reach the famous Everglades. If you fail, you may be in very deep trouble.

My youngest son and keen assistant, Christopher, with a beautiful (and heavy) lady of *L. polyphemus.* (Plantation Key, Florida)

On the western coast of Florida you may find discrete populations all the way up north, including Marco Island, Estero Bay, Sanibel Island, Charlotte Harbor, Sarasota Bay and Saint Joseph's Bay in the northwestern part of the panhandle.

The North American Indians knew the horseshoe crabs well, too, on the coast of Virginia, for instance, and particularly in Chesapeake Bay where horseshoe crabs still abound.

The State of Virginia, so called after Elizabeth, the Virgin Queen, was discovered, annexed and named by Sir Walter Raleigh in 1584. On April 9 of the following year, a small fleet of 5 settler ships left the shores of England for America, and under the command of Sir Walter's cousin, the capable Admiral Richard Grenville, they reached the coast of Virginia safely on August 17.

The passengers included the painter John White and the historian Thomas Hariot, who were also both competent natural historians. It was their job, among other tasks, to help the settlers distinguish between useful and useless—and possibly harmful or dangerous—plants and animals in the New World.

When they had made contact with the Indians, they both noticed that the local population caught fish on spears whose tips were neither bone, stone nor metal. They were the tails of a strange "fish" which the Indians called *Se-ekanauk.*

White and Hariot produced copious reports on this use of the rigid, hollow and quite harmless horseshoe crab telson. 75 of White's original drawings are now in the British Museum, collected under the idiomatic title "The Pictures of Sundry Things Collected and Counterfeited According to the Truth, in the Voyage Made by Sir W. Raleigh, Knight, for the Discovery of La Virginea, in the 27th Year of the Most Happie Reigne of Our Soveraigne Lady Quene Elizabeth".

A female *L. polyphemus* heading for the beach and the waiting males during the breeding season.
(Lignumvitae Key, Florida)

A mating couple of *Limulus polyphemus* off Lignumvitae Key. Note the comparatively small size of the attached male.

A mating pair of *L. polyphemus.* The male is trying hard to turn the couple back again by means of its telson.
(Lignumvitae Key, Florida)

Hariot's description of the *Se-ekanauk* and of the Indian fishing spears was published in London in 1588: "A Briefe and True Report of the New Found Land of Virginia". He also described the Indian custom of eating the meat and keeping the large concave shields of the horseshoe crabs once their telson had been broken off. The shield, or carapace, had a hard surface which made it a durable vessel for food and drink.

The settlers thought of many different nicknames for the horseshoe crab, and they all referred to the shape of its characteristic carapace. One name stuck: Horseshoe crab and today, all four species of horseshoe crabs are known popularly under this very apt name.

Horseshoe crabs abounded along the shores of the American east coast, so the settlers began using dried, crushed Aristocrats as fertilizer on their fields and as food for pigs and poultry.

In 1856, more than 1.2 million animals were caught for these two purposes along just a few kilometres of the Cape May beach in Delaware Bay. The take culminated in 1930, when no less than 5 million horseshoe crabs were killed along the entire east coast of the USA. Better types of fertilizer were gradually introduced, and a diet of horseshoe crabs gave an unfortunate (?) after-taste to meat and eggs, so eventually the horseshoe crabs were again left alone.

Spawning time varies somewhat latitudinally, but generally it peaks in May and June. When the sexually mature adults head for the breeding beach, the males will arrive first, patrolling along the foot of the beach awaiting the females, which in due time move directly to the beach. The likelihood of a female reaching the beach unattended is very hypothetical as the number of males involved is often several times the number of females. Up to six males queuing up behind a single female and attached to each other's opisthosoma by means of the fistlike grasping claws has been observed by the author on Marquesas Keys. On that occasion the males clearly employed their strong telsons as weapons to ward off competitors in the queue of wooers.

A mating couple of *L. Polyphemus* has been caught by the low tide during the egg laying procedure. The female has died and the male is apparently too weak to make its way the few metres back into the water.
(Marquesas Keys, Florida)

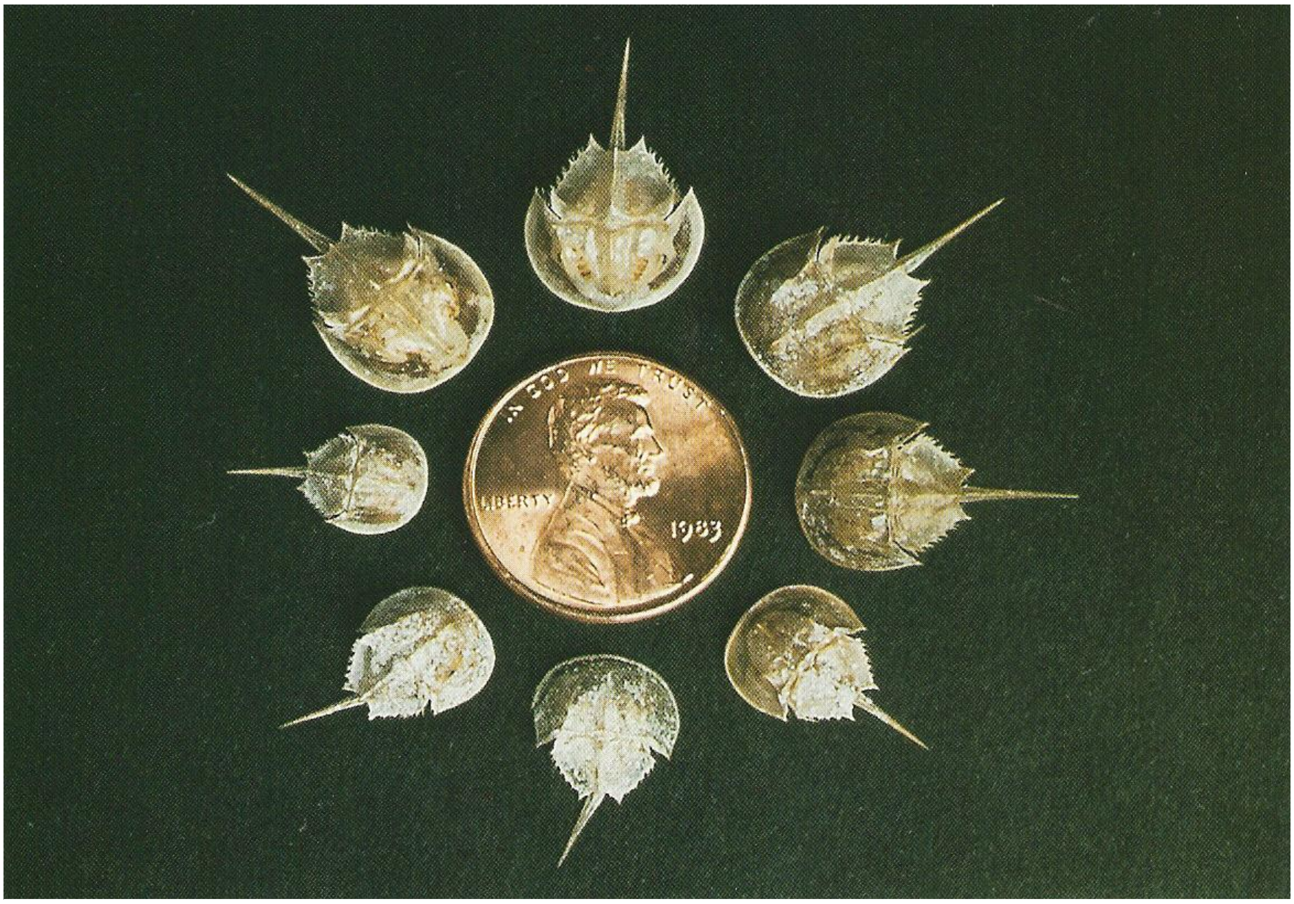
Spawning occurs at high tide usually on estuarine beaches that are at least partially protected from the force of the oceanic waves. Low energy beaches within bays and coves are ideal locations for spawning and larger spawning groups will assemble only during periods of calm or relatively calm weather.

Eggs are deposited between the low and high water marks of a spring high tide in "nests", in clusters on the beach at a depth of approximately five to seven inches. Each female carries about 88 000 eggs per year, and, on average, one may find about 3 650 eggs with a diameter of 1.6 to 1.8 mm when newly laid in each nest.

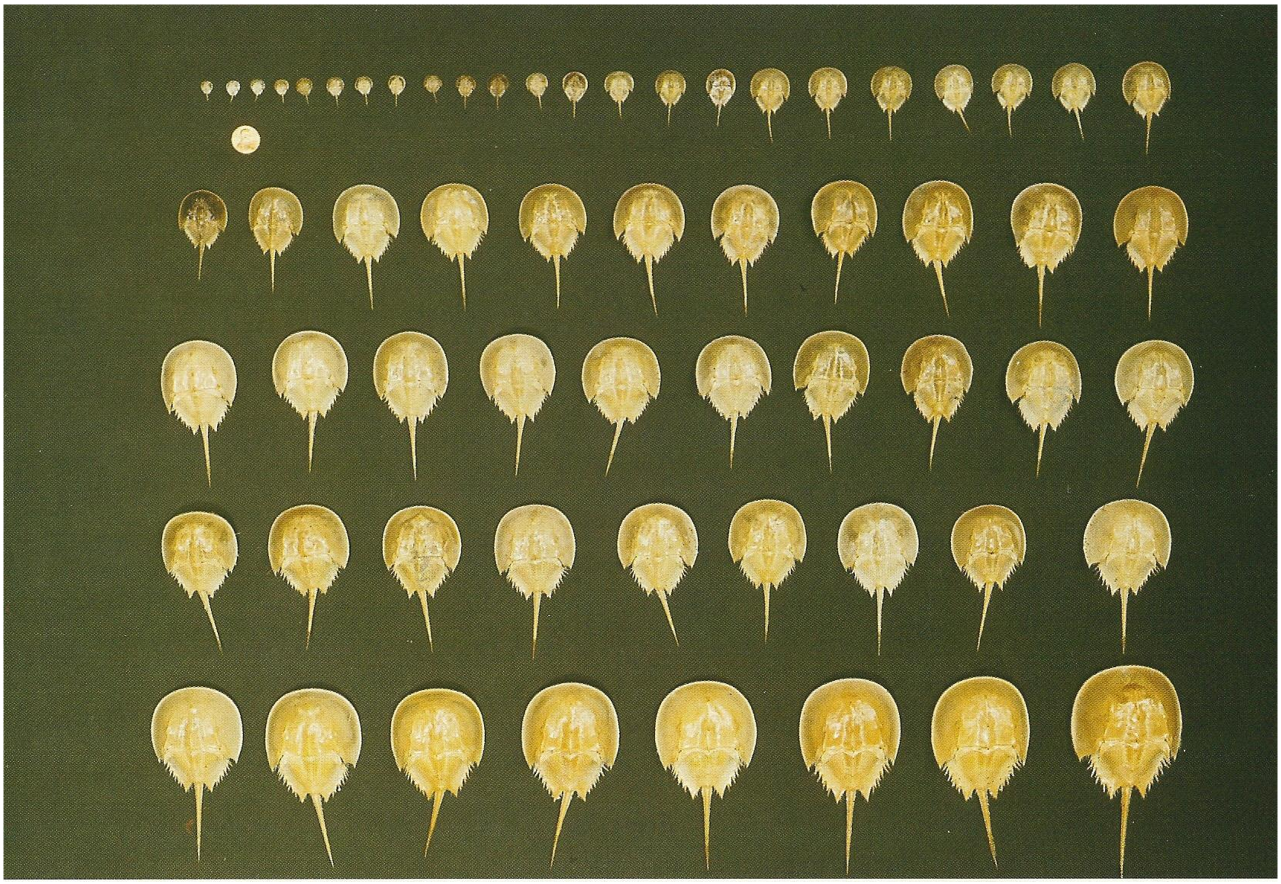
Since the microclimate (a combination of temperature, moisture and oxygen) is of very significant importance to the development of the eggs, the distribution of nests on a beach differs geographically depending, at least in part, upon the amplitudes of the tide.

Early in the embryonic development the chorion ruptures and a new membrane that has been secreted by the embryo enlarges to form a transparent, spherical capsule. After the fourth intermoult, when the trilobite larva emerges from the egg capsule, which ruptures as a result of age and larval activity, we have a free swimming trilobite larva with still no telson and an average size of 3.1 mm. The trilobite larvae still carry some yolk and do not seem to have to eat. They swim actively by flapping their gills while lying ventral side up, and a couple of weeks after emerging from the egg they settle down to moult. Despite the possibility of fairly wide dispersion during their free-swimming period, most larvae settle in shallow water, often intertidal areas, on or near the beaches where spawning occurred.

Following this moult they acquire a telson and spend more time walking with their dorsal side up, just as they begin to burrow in the substratum.



Part of the author's collection of sheds from *L. polyphemus.* The one cent coin (diameter: 18 mm) has been included for comparison of size.



Sheds from *L. polyphemus,* all collected in the same area of Florida Keys. The one cent coin
In the upper left hand corner serves as a measure.

First tailed-stage juveniles appear to be devoid of yolk and begin to eat, accepting a variety of food such as bits of nereis and mytilus, frozen brine shrimp, marine nematodes and small polychaetes. At a size of approximately 4.5mm during the first juvenile tailed-stage the horseshoe crab is translucent. In the second juvenile stage the short, distinct telson grows faster than the rest of the body, and some brown pigmentation appears in the carapace. The time from fertilization to hatching in *L. polyphemus* is about 3 to 5 weeks depending on the locality.

The young limuli undergo ecdysis six times during their first year of life, each time developing a new, smooth carapace devoid of ectocommensals and each time growing about 25% in linear dimensions. They spend their early childhood all the year round on intertidal mud or sand flats adjacent to the breeding beach, burrowing into the substratum at low tide (and during turbulent weather) and foraging during high tide, quickly learning to probe through the substratum with the chemosensitive chelae of the prosomal appendages in order to capture small polychaetes and other food items by rapid excavation and pinching movements.

The exuviae and new shells of young animals are fragile and transparent, but they become darker and heavier with each successive moult. This juvenile fragility exposes the young crabs to the predation of a variety of enemies, and it certainly takes a lot of resourcefulness and good luck to survive the first couple of years. Several species of nematodes and oligochaetes and maggots of flies abound in "old" horseshoe crab nests, apparently they feed on egg membranes as well as larval limuli.

The sand shrimp feed upon horseshoe crab eggs, just as fiddler crabs have been observed to feed on the first - and second - tailed stages of *L. polyphemus.* At Barnstable harbor, Massachusetts, a blue crab, a green crab and an unidentified species of spider crab were observed to attack immature limuli, especially while the latter were moulting.

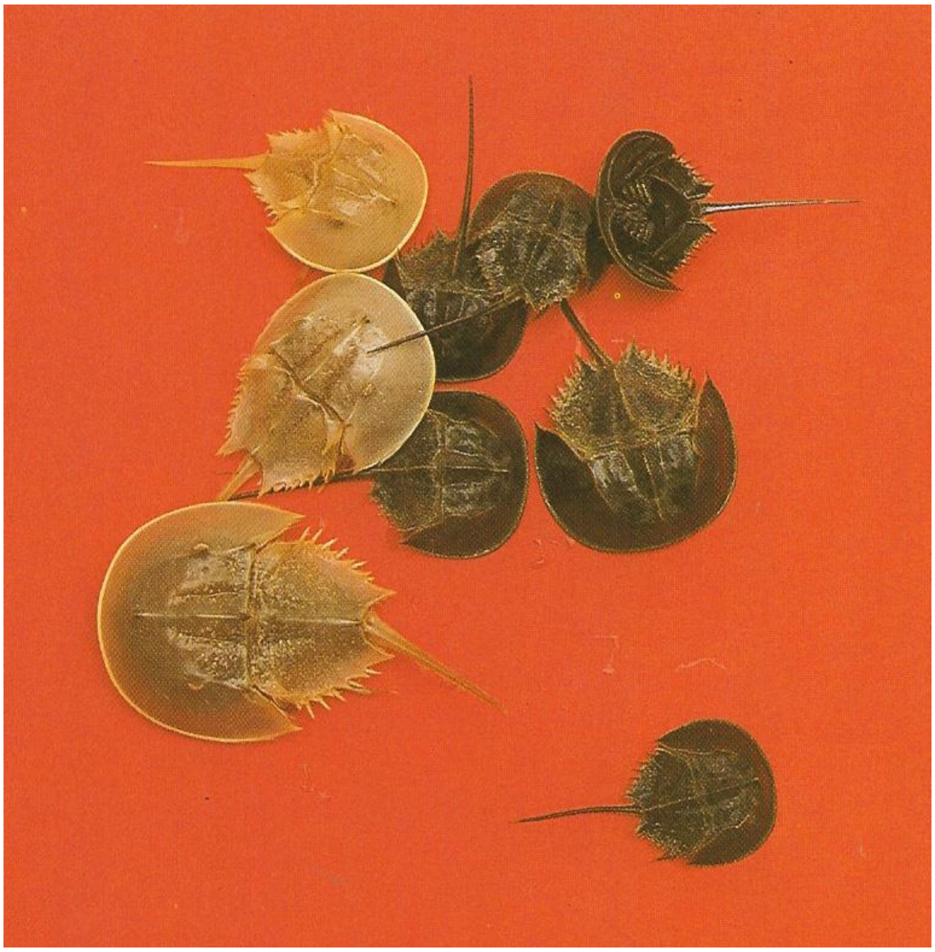
In the tidal streams and shore zones of estuarine systems, horseshoe crab eggs are much appreciated by striped bass and white perch, and eggs and larvae are also eaten by a variety of other fishes including striped killifish, silver perch, northern kingfish, Atlantic silverside and flounders.

Eggs and larvae are also eaten by eels working their way methodically alongside spawning limuli, just as large schools of mullets have been seen feeding upon eggs and larvae of *polyphemus* washed from the beaches during high tide.

Puffers will attack the underside of half grown limuli, biting off their legs and gill books, and catfish by the hundreds have been observed surrounding limuli spawning on a sandy flat near the mouth of the Caloosahatchee river, north of Sanibel Island, Florida, voraciously devouring the eggs as fast as they were laid.

As if these threats from the sea were not enough, plovers, sandpipers and gulls will also prey on *Limulus* eggs and larvae in the beach nests and adjacent intertidal flats, and, in Florida at least, even the pelicans will eat their share of the young horseshoe crabs.

I do not wish to encourage further nest robbing, but those who would like to study *Limulus* embryology in more detail may obtain fertilized eggs in two ways: During the spawning which generally occurs in late May and early June and peaks around the full moon, nests can be located on favorite spawning beaches in the morning following a spring tide. The eggs are deposited near the high tide line, so before the next high tide it should not be too difficult to find the nests and dig for eggs. Outside of the normal spawning period (mid-April to mid-August) viable gametes can be obtained from adults, and the eggs fertilized *in vitro.* Gentle pressure anterior to the genital flap is usually sufficient to release mature ova or sperm. The rate of development varies greatly among individual eggs in a single clutch and is strongly temperature dependent, but pooling eggs or sperm from several animals has no detectable effect on development.



Juveniles of the Chinese species *Tachypleus tridentatus* and the American species *Limulus polyphemus* meet in Copenhagen, Denmark.

Juveniles of the Chinese species *Tachypleus tridentatus* and the American species *Limulus polyphemus* meet in Copenhagen, Demmark.

During their ontogeny horseshoe crabs will consecutively occupy a series of habitats according to a general but typical pattern that may be aligned with gradients such as salinity and water depths.

As the young limuli grow older they move away gradually from their shallow natal area into deeper estuarine waters, often to sandier substrata, although they may return to the mud flats to forage during high tide.

Adults are found in major concentrations at depths less than 10 metres, although some specimens have been dredged off the mid-Atlantic portion of the continental shelf at depths up to 246 metres. The adults remain offshore for the duration of the life cycle, returning to the (natal?) beach only to breed.

Horseshoe crabs are slow-growing animals and their development to sexual maturity requires 9-10 years for males and 10-11 years for the females. Both sexes have a life expectancy of at least 15-18 years in the northern part of the range. After the first year, limuli will moult only once a year until they reach sexual maturity, when moulting is believed to stop altogether.

The carapace of limuli, particularly adults, is a suitable habitat for a number of species, and the frequency of moulting is probably a deterrent to sustained attachment of ecto commensals and epiphytes. These sometimes develop into veritable zoological gardens and are most prevalent on adult limuli because they do not moult as frequently (if at all), permitting continued individual growth as well as population development of the attached organisms, which may include algae and protozoa, but certainly also commensals like coelenterates, bryozoans, annelida, molluscs and tunicates.

A juvenile *L. polyphemus* carrying its "old self" on its back - minutes after the molting process is finished.

(Islamorada, Florida)

One busy morning of beachcombing around Plantation Key may result in a nice collection of sheds.

It is believed that the color of the adult carapace may be related to substratum color and water turbidity. Thus Atlantic coast limuli, north of the lighter-colored sands and less turbid waters of Florida, tend to have dark-colored (brown and green - even black) carapaces, while limuli along the east and west coast of Florida are much lighter colored (browns and greens). This rule, however, is not without exception.

Reaching maturity and acquiring a hard carapace are no guarantee against all predators, however, although the enemies are by now certainly more impressive. 40 horseshoe crabs were found in the stomach of a 12-foot tiger shark caught off Sarasota, Florida, and, likewise in Florida waters, over a bushel of adult limuli were found in a leopard shark. Turtles, especially the big loggerhead turtles, enjoy tearing out the gill books of adult crabs, and devil rays and swordfish have also been known to attack limuli.

During its normal lifetime a horseshoe crab is likely to encounter a variety of environmental challenges. Although the majority of horseshoe crabs live at normal marine salinities, juveniles in intertidal areas and adults entering estuarine systems to breed encounter lowered salinities, just as juveniles, in particular, and adults to a lesser extent occasionally experience sharply reduced availability of oxygen, moisture and food,

Depending on to their geographical range, the horseshoe crabs also encounter a very broad temperature range, and spending a winter on the mud flats or being stranded during Low tide in the case of breeding adults, call for quite impressive powers of adaptation.

It is hardly likely that more than one species of *Limulus* exist, but considering the above mentioned different conditions of water temperature, salinity, cold, etc. it would not be surprising if *physiological races* of *Limulus* exist.

The American *Limulus polyphemus* is distinguishable from the three Oriental species by a number of characteristics, as would reasonably be expected concerning species inhabiting areas so far apart.

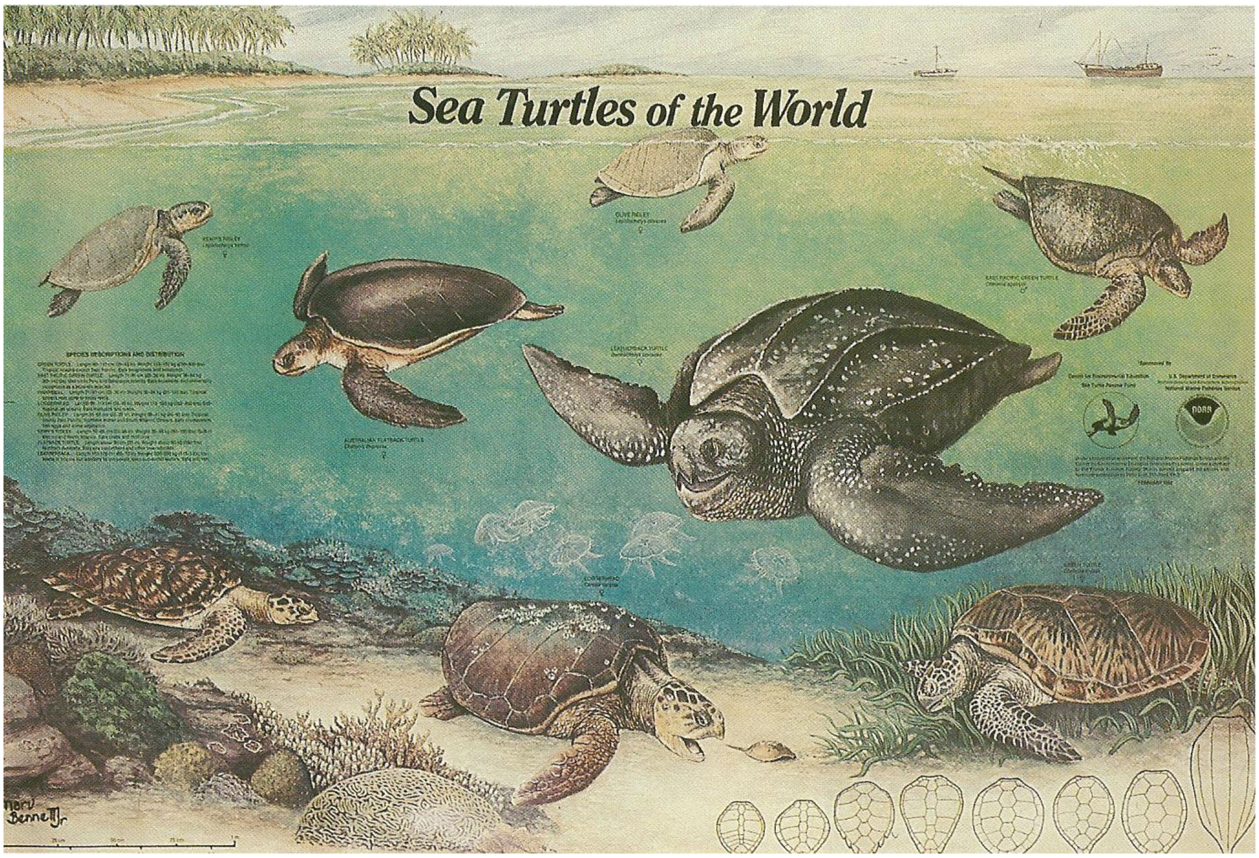
A closer examination of the extant Oriental species reveals that while agreeing with one mother in the main, *Tachypleus gigas* and *Tachypleus tridentatus* are more closely related to each other than either is to *Carcinoscorpius rotundicauda,* and all these three are then more closely related to each other than to *L. polyphemus,* as mentioned above. Such seductions from morphological and phylogenetic studies are supported to a certain extent by serological analysis and hybridization experiments.

Dorsal view of a large female *L. polyphemus* and the small male. Note the distinct difference in color.

(Lignumvitae Key, Florida)

Ventral view of the same pair of *L. polyphemus.* The coloration of the female is much lighter, although they were caught in the exact same area.

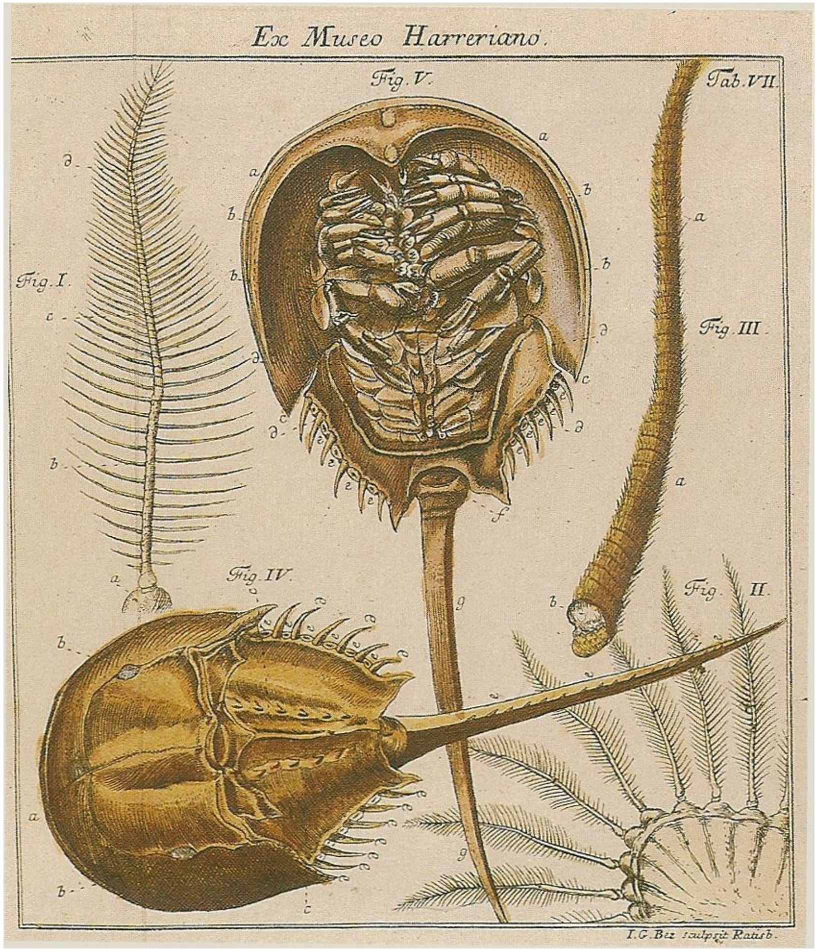
A nurse shark and a loggerhead turtle side by side. Both are known to savour horseshoe crabs occasionally.
(Plantation Key, Florida)



An American poster offering information about some of our endangered turtles and illustrating the fact that turtles enjoy eating the soft gill books of horseshoe crabs.

Sharks are among the very few natural enemies of adult horseshoe crabs. This nurse shark was photographed during mating just off the Marquesas Keys in the Gulf of Mexico.

A female *L. polyphemus* on her way into our net. This grassy mudflat is situated three miles off the Florida Keys, in the Gulf of Mexico.



Ancient manuscript drawing of a male (fig. V) and a female (fig. IV) of *Limulus polyphemus.*

Discussions about taxonomy usually focus on certain easily recognizable characteristics that suffice to diagnose the various taxa, while more general discussions tend, quite naturally, to focus on the high degree of similarity among the extant horseshoe crab species. If you have ever held a horseshoe crab in your hands (they are absolutely harmless despite their somewhat threatening appearance), you will immediately and without slightest hesitation be able to identify any horseshoe crab species anywhere in the world as at least a horseshoe crab, whether it is a giant Chinese *T. tridentatus* weighing 7.9 kilograms or a juvenile American specimen a few inches long.

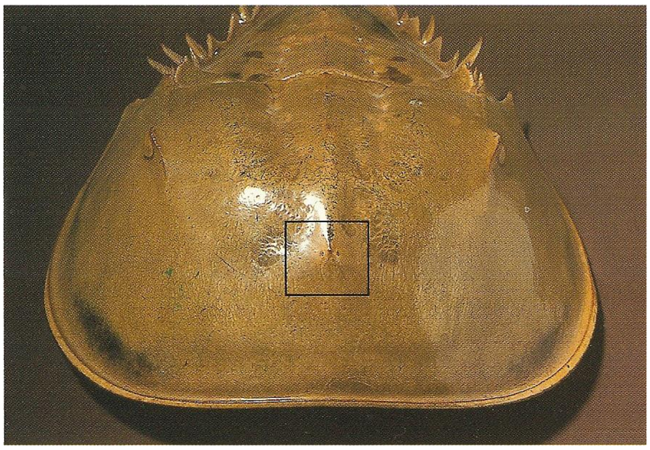
The prosoma of horseshoe crabs is semi-circular (or horseshoe shaped) in outline and the appendages lie wholly within the ventral cavity formed by the more or less steeply sloping sides of the prosoma (the Oriental species are all somewhat flatter than *Limulus polyphemus).*

The exoskeleton is of tough chitin, generally smoothly polished and kept free from ectocommensals and epiphytes by means of a glycoprotein exudate produced by hypodermal glands and secreted through the carapace. This exudate has antimicrobial properties and also serves as a mechanical barrier to potential pathogens.

The dorsal side of the prosoma is furnished with seven spines. One of these near the fore-edge is just behind the two small median ocelli. Across the middle is a row of three spines and at the bases of the outside ones lie the lateral eyes, staring outward. The posterior margin of the prosoma is a three sided re-entrant with a short, erect spine in the middle and one at each corner. These dorsal spines are especially prominent in immature horseshoe crabs, and they diminish gradually and proportionately with the increasing size of the animal. These spines are believed to help small animals to gain a better purchase during burrowing (since all the fixed spines point slightly backward), and also to render the young animals less inviting to predators because of the sharpness of the spines.

The concave ventral side of the cephalothorax contains six pairs of appendages. The chelicerae are small chelate organs of three segments, the chelae are smooth and finely pointed. They are situated centrally, just in front of the mouth, and serve as knife and fork (or chop-sticks). The next five pairs of legs are all composed of six segments, and the proximal joints, next to the mouth, carry a masticating gnathobase on the inner side.

The sixth pair of prosomal appendages is characteristically modified in each species. The tibia which is fringed with hairs carries four leaf-like processes at its distal end on the side of the tarsus which is itself forked. When the legs are drawn forward these appendages lie closely together, but when the legs are thrust back the resistance of the water (or the substratum) opens them fan wise and they form a very useful scoop with which the animal pushes away the sand or mud as it buries itself in the sea floor.



Frontal view of a male *L. polyphemus.*

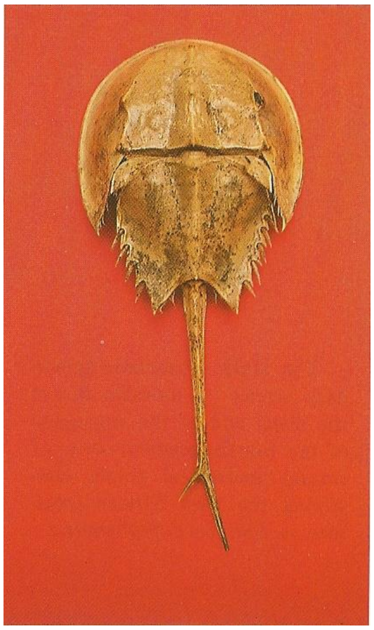
A close-up view of the median ocelli in *L. polyphemus.*

A female *L. polyphemus* preparing to dig into the bottom off the Marquesas Keys.

A close-up ventral view of a female *L. polyphemus.* (Cotton Key, Florida)

Ventral view of a female *L. polyphemus* in momentary distress.

(Lignumvitae Key, Florida)



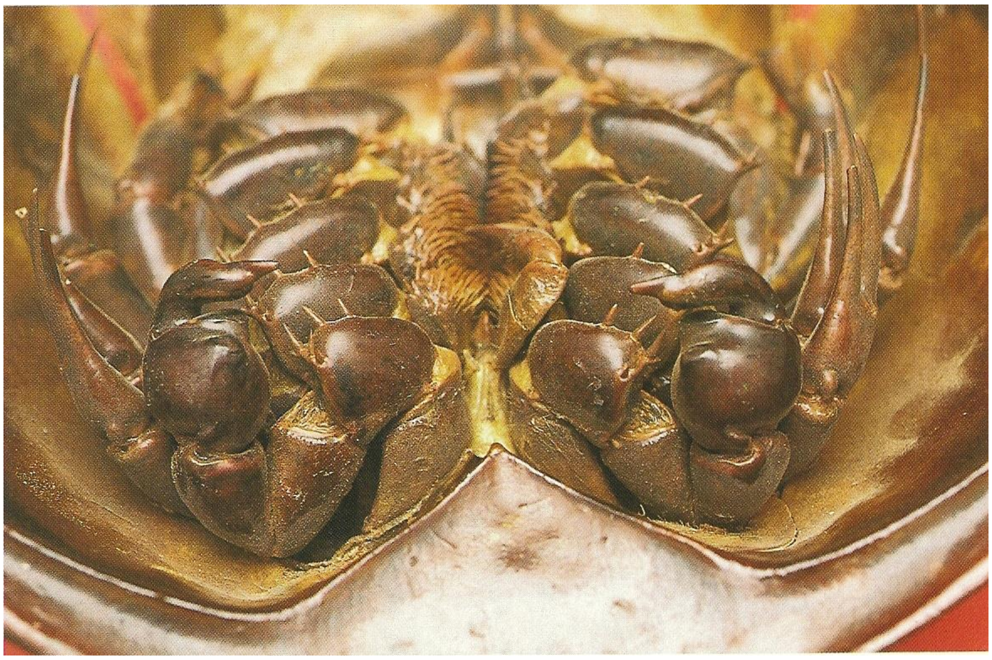
A juvenile *L. polyphemus* with a forked tail - one of the more frequent anomalies found in horseshoe crabs.
(Plantation Key, Florida)

A female *L. polyphemus* photographed at 2 a. m. off Cotton Key in the Gulf of Mexico.

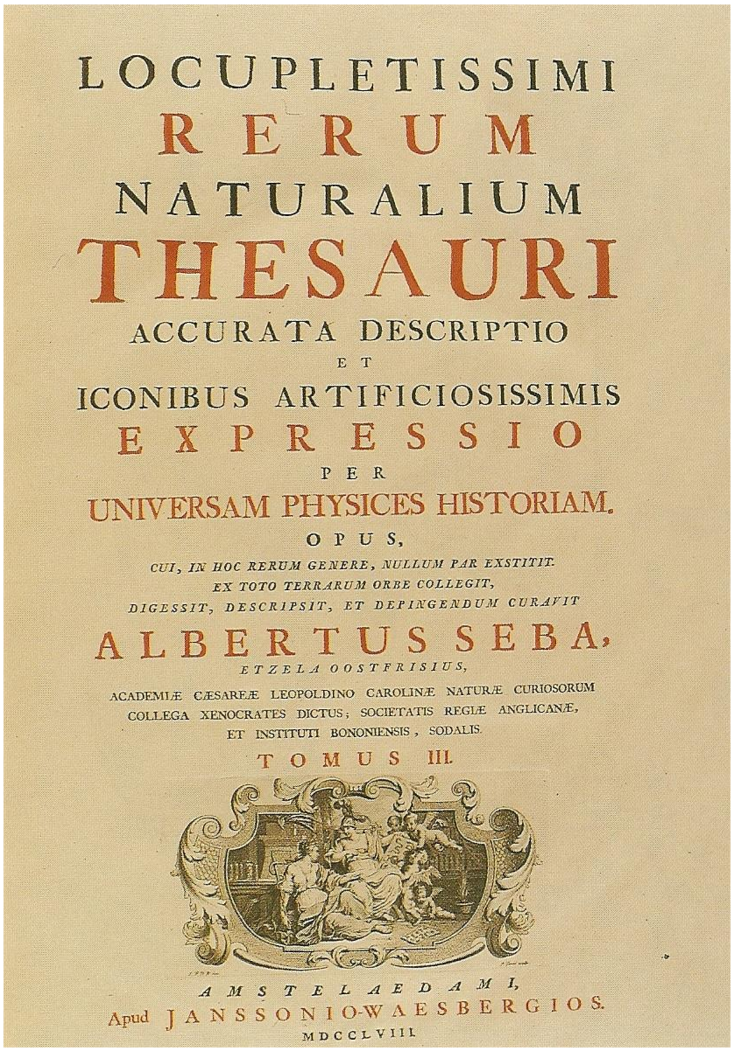
Behind the mouth is an apparent seventh pair of appendages, the chilaria. These are small round plates, joined together at the base, whose function is not known. Morphologically they do not seem to be true appendages, but to be homologueous with the pentagonal sternal plates of the terrestrial relatives, the scorpions.

The opisthosoma fits into the three-sided re-entrant of the prosoma mentioned above. It is a broad hexagon with three spines along its middle line. Its postern lateral margins carry six small spines and terminate in a seventh much larger one. Between these, six moveable spines are articulated, varying in size depending on the species and sex of the individual horseshoe crab.

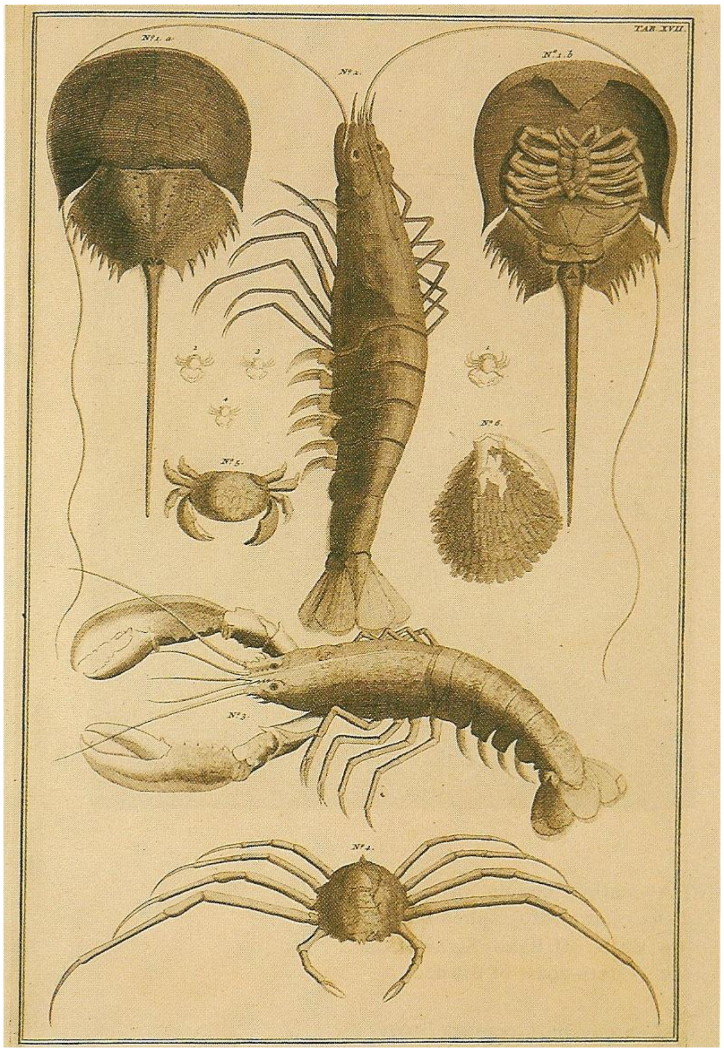
The sixth prosomatic appendage carries a movable spur at the distal end of the underside of the fourth segment - one of several anatomical details classifying the west African specimen as *Limulus polyphemus.*



Ventral view of a male *L. polyphemus.* Note the fistlike grasping claws and the spiny appendages. This specimen was allegedly caught by Korean fishermen off the west coast of Africa.



Thesaurus from 1758 ……..



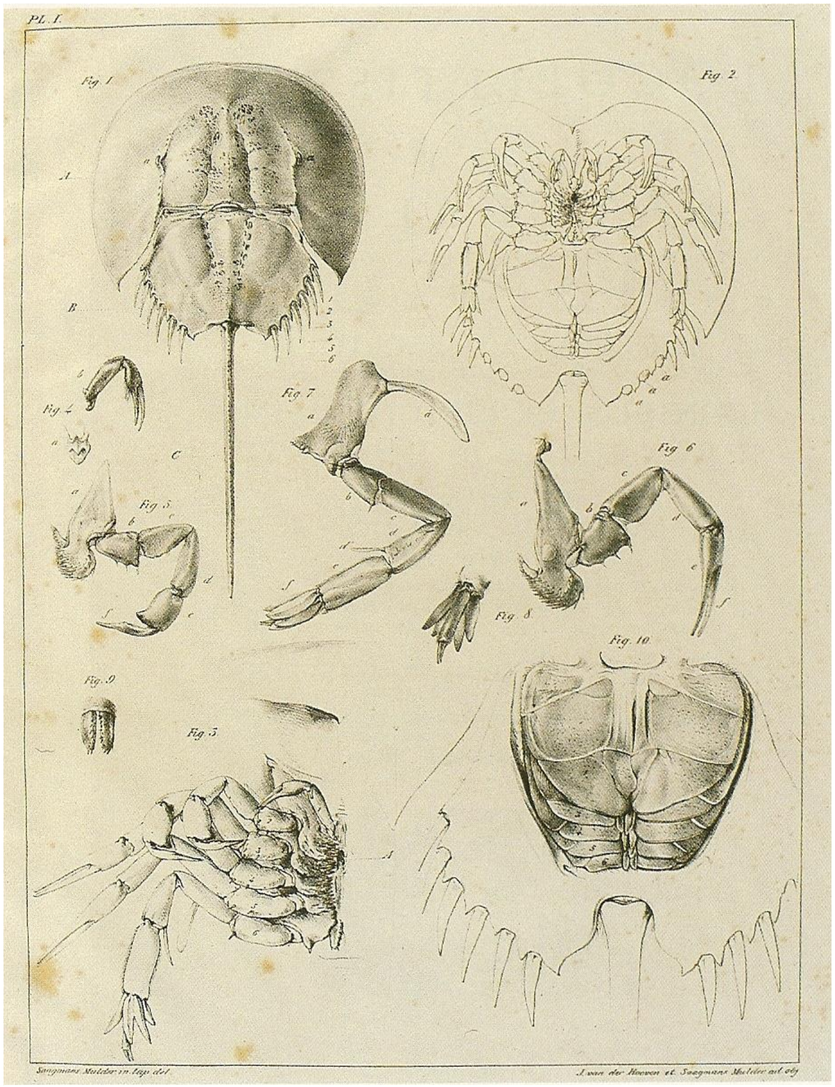
….. depicting a pair of *L. polyphemus.*

The posterior margin of the opisthosoma is a three-sided concavity similar to but smaller than that of the prosoma. Articulated from its central edge it carries the strong, pointed spear known as the telson, which is almost as long as the rest of the animal. Depending on the species, the telson may be triangular or cylindrical in section, crested or non-crested on top, lightly convex, concave or flattish at the base. The telson is used by the horseshoe crab as a means of righting itself when it falls upon its back, and it is dragged or thrust into the substratum behind when the animal moves forward.

One of the chief characteristics of horseshoe crabs is the existence of six mesosomatic appendages lying well protected under the opisthosoma. The first is known as the genital operculum since below it lie the genital pores. In the male the genital pores are on the apex of a firm conical projection, while in the female the pores are broad slits or orifices in soft convex areas. The genital operculum is larger than the following appendages and may overlap the first one or two of them. The five appendages following the genital operculum are similarly shaped plates which lie regularly overlapping each other, but which can be opened and move to and fro as is particularly visible when a horseshoe crab is occasionally seen swimming ventral side up.

These appendages carry the respiratory organ, or gill-books, ten in all, each containing between one hundred and two hundred leaves in which the blood flows.

Males can usually be distinguished from females by their smaller size and by their shorter, stouter pedipalpi.



Limulus moluccanus = Tachypleus gigas. The male (Fig.1) and female (Fig.2) of *T. gigas* supplemented with anatomical details on a beautiful manuscript drawing from 1838.

(J. van der Hoeven)

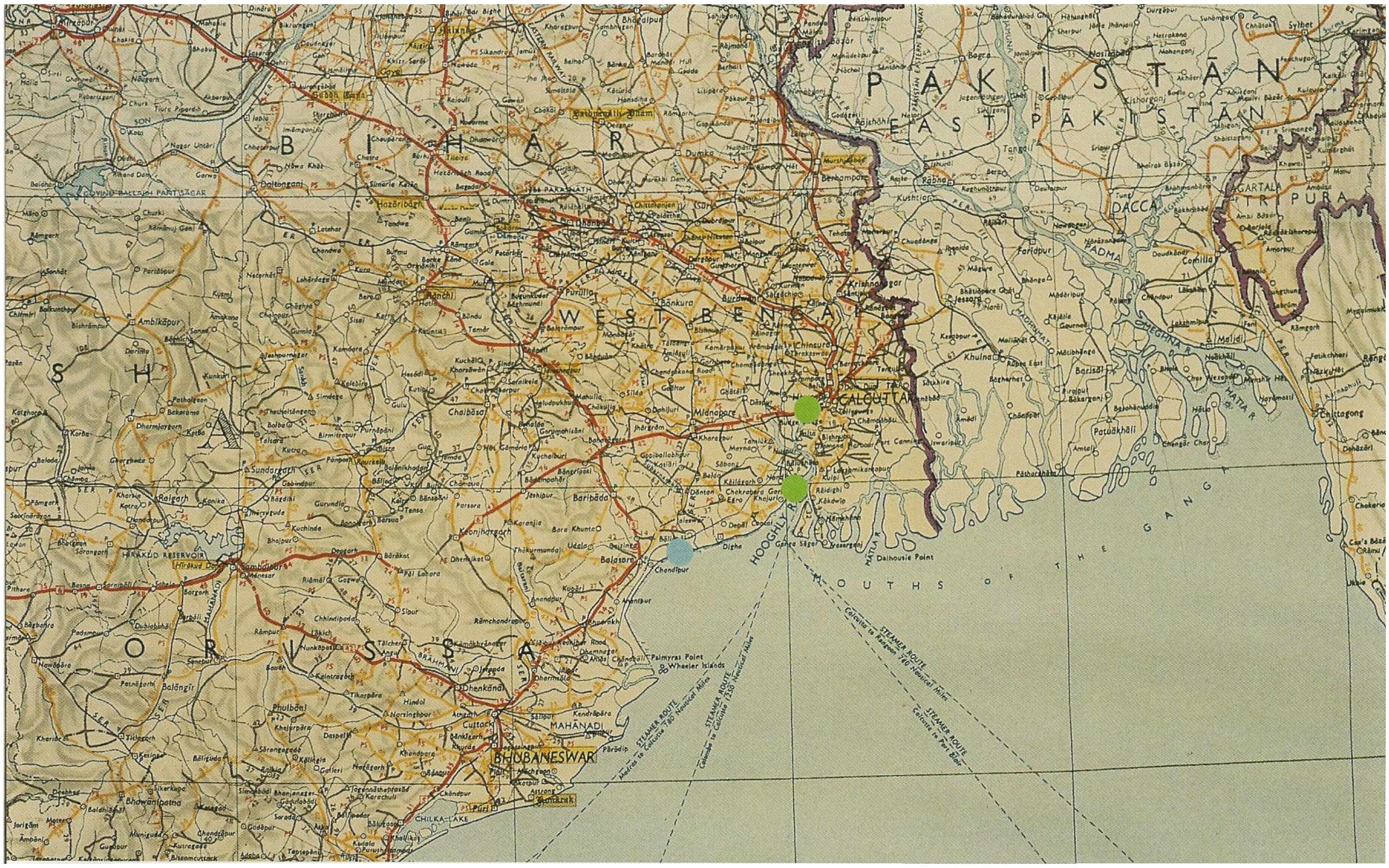
A mating couple of *Tachypleus gigas* photographed in shallow water off Bako, Sarawak on the western coast of Borneo.

In the development of secondary sexual characteristics the American species is less specialized than the Oriental relatives. In *Limulus polyphemus* only the tarsi of the anterior legs are modified as claspers to grasp the female, and there is no variation in the lateral, moveable spines of the opisthosoma of the female, while in the Indo-Pacific species both the second and third pairs of prosomal appendages are modified into holders in the male, and the three posterior spines of the adult female are markedly shorter than the anterior ones, especially in *Tachypleus.*

Carcinoscorpius rotundicauda differs both in this respect and in the chelate condition of the claspers in the male, a peculiarity which is believed to have preceded the hemichelate condition of these appendages observable in *Limulus* and *Tachypleus.*

Generally the prosomal subfrontal arch of the male is higher than that of the females.

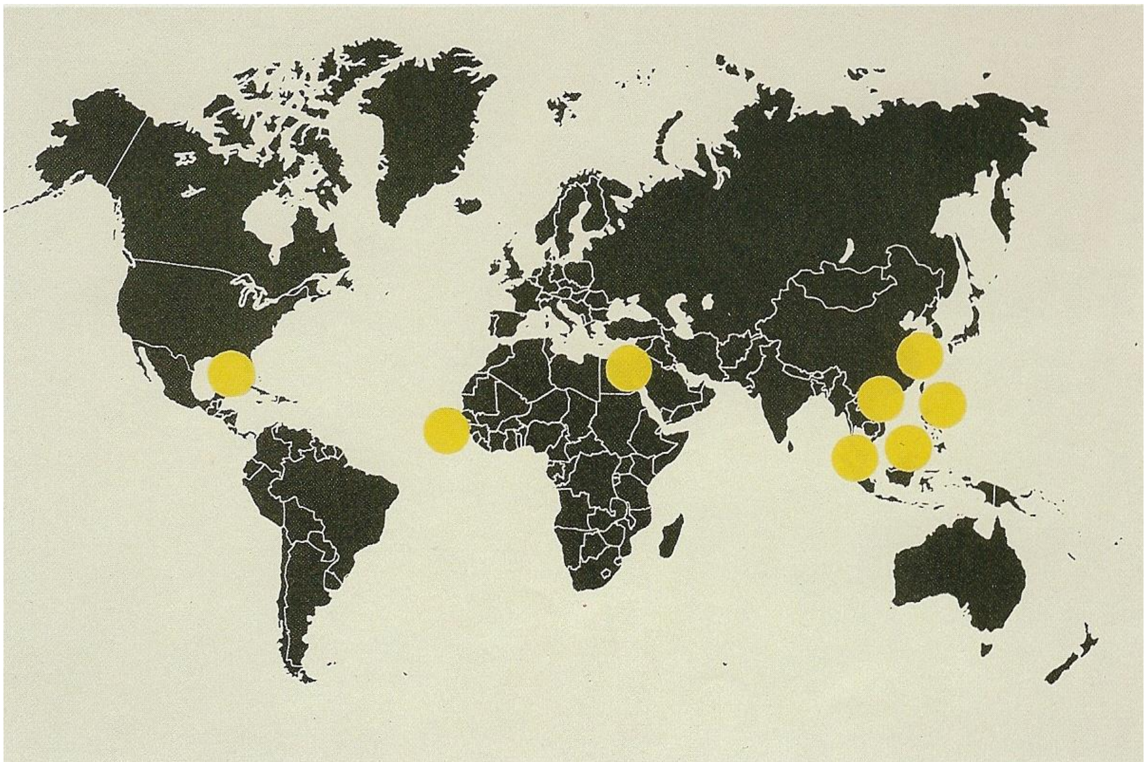
Apart from the general *Xiphosuran* features outlined above *Limulus polyphemus* is characterised by having a movable spur at the distal end of the underside of the fourth segment of the sixth prosomatic appendage. The postanal spine or telson is triangular in section, carrinate at the top and lightly convex or flattish at the base. The clasper of the second prosomal appendage of the male is hemichelate, the immovable digit reduced to a short, thick thumb-like prominence.



The habitats of Indian species of *Carcinoscorpius rotundicauda* and *Tachypleus gigas:* The Bay of Bengal and the huge estuary of the river Ganges.

T. gigas

C. rotundicauda



Areas covered by the author's expeditions 1978-1986 including 3 to the United States of America and the Gulf of Mexico, 7 to China, 4 to Africa and the Middle East and others to Southeast Asia, especially Malaysia., Sarawak and Sabah on Borneo and the Philippines.

Tachypleus gigas

Tachypleus gigas may still be known as the *Indo-Papuan King-Crab,* although "king-crab" is a misnomer, since *Paralithoides camtschatica,* a crustacean, is the true king crab.

Tachypleus gigas has a wide geographical range with its westernmost distribution along the Indian Orissa coast in the northern part of the Bay of Bengal. *T. gigas* can be found near Chandipore (Balasore District, Orissa) in the vicinity of Burhabalang River, close to its fall into the sea. This species is also known to migrate far into the Ganges estuary, as far north as the brackish waters around Port Canning in the Sunderban area southeast of Calcutta, where 53 ripe females have been collected in the month of May (Dr. Anil Chatterji, personal communication).
T. gigas occurs discontinually along both coastlines of the Malay Peninsula and around Singapore and outlying islands.

I have seen fairly numerous populations from Port Dickson to Melaka on the west coast, around Singapore Island, and on the east coast of Malaysia where *T. gigas* is abundant in estuarine areas near the cities of Kuantan and Mersing (whereas no horseshoe crabs were found on the outlying islands: Pulau Rawa and Pulau Tioman).
On the southeastern tip of the Malay Peninsula due east of Singapore, *T. gigas* is very numerous from Desaru south to Kampong Pengerang, where the biggest specimen of *T. gigas* I have ever found was picked up on a spawning beach near the Kampong. It measured 45.5 cm in total length (including the telson) and the prosomal width was 22.0 cm. On this expedition through Southeast Asia we measured altogether 506 specimens of *T. gigas.*
T. gigas is also known from Java in Indonesia, and it may presumably still be found on the northwestern shores of the island, near Tandjung Sadari, where Iwanoff observed it in 1906.

On beautiful Borneo *T. gigas* lives (discontinually, as usual) on the western and northern coastlines from, at least, Kuching (Sarawak) to Kudat (Sabah) and with a numerous population centered around Bintulu, and a smaller, but still substantial presence in the vicinity of Miri, just south of Sarawak's border to Brunei. During the breeding season *T. gigas* in great numbers can be encountered in Datu Bay, east and west of Kuching, and, quite easily, you may see (but not touch) the species at Bako National Park facing Santubong Island.

Fishing boat returning to the wharf near Mersing, Johor, Malaysia.

Crystal clear, clean water surrounds Pulau Tioman - the very beautiful island three hours by motor launch northeast of Mersing, Malaysia.

Moving north again, *T. gigas* inhabits the Gulf of Siam and can be bought in the central fish market in Bangkok. If you want to collect your Thai specimens yourself, this can be done in several places not far from Bangkok, since the animals sold on the fish market are supposed to be collected at the mouth of a nearby river, Chao Phraya, the River of Kings.

Continuing the trail eastwards, we meet *T. gigas* in the northern part of Viet Nam and in the Beibu Bay (the Chinese part).

According to my observations, the northernmost distribution of *T. gigas* is Deep Water Bay in Hong Kong where it overlaps with *Tachypleus tridentatus.*

Earlier reports have indicated that *T. gigas* lives in the Molucca Sea (around Manado, on the northern tip of Sulawesi) and in the Halmahera Sea (on the coast of the island of Halmahera), but this has not been confirmed by the presentation of live animals in recent time. In my collection I have a preserved specimen of *T. gigas* donated to me by Korean fishermen who allegedly had brought it with them alive from the Moluccas.

A specimen from the Torres Strait between Australia and New Guinea is kept at the British Museum, and I have recently been informed by an Australian in Hong Kong that horseshoe crabs are found on the Australian coasts of Cape York Peninsula and Queensland. It would be of great interest to have this possible Australian species identified and its habitat located more precisely.

Finally, *T. gigas* has been reported from the Philippines by Willemoes-Suhm in 1883 and Pocock in 1902, but personally I have not been able to locate *T. gigas* in the Philippines or obtain any pertinent information about it - can anybody help?

A glimpse of the fascinating coral world surrounding Pulau Tioman, Malaysia.

Tachypleus gigas is essentially a marine species occurring on sandy beaches and muddy bottoms from the tide-line to depths exceeding 20 fathoms.

It was commonly known as *Limulus moluccanus* (Latreille), or even earlier by the name *heterodactylus* (also Latreille) until Milner gave the species its final and present name.

From the limited experience of having examined approximately one thousand specimens of *T. gigas,* it is my impression that this species is more often subject to exoskeletal abnormalities, due to the regrowth of injured parts of the carapace and/or the appendages, than any other horseshoe crab species.

Latreille proposed a new species named *Limulus virescens* based upon a specimen which had seven rounded conical and pointed sclerites at the base of the penultimate segment of the sixth prosomatic appendage instead of the four of the normal flattened form.

In 1902, Pocock introduced what he believed to be a new *species, Tachypleus hoeveni,* based upon a Moluccan horseshoe crab specimen described and drawn by the Dutch scientist J. van der Hoeven in 1838. According to its definition, *T. hoeveni* resembles *T. gigas* in all known respects except that the median terminal segments of the genital operculum in *hoeveni* are separate and overlap asymmetrically, instead of being united, symmetrical and contiguous as in *T gigas.*

Prior to van der Hoeven's publication in 1838, horseshoe crabs from the Moluccas had been described by at least three authors.

In 1558, Joannes Jonstonius published his book *Historia Animalum* in which he depicted *Cancer mollucensis* based upon a specimen believed to have been brought back from the Moluccas.

In 1605, the Dutch scientist Carolus Clusius published *Exoticorum Libri Decem* containing a drawing of another horseshoe crab also allegedly brought back to Holland from the Dutch East Indies.

And finally, one hundred years later, in 1705, the famous work *D' Amboinische Rariteitkamer* by Georgius Everhardus Rumphius was published in Amsterdam depicting *Cancer perversus.* Since Rumphius lived and worked on the Moluccan island of Amboina (Ambon) his information and illustrations must be considered authentic. Rumphius also mentioned that he received a horseshoe crab specimen caught near the city of Manado on the northernmost tip of Sulawesi.

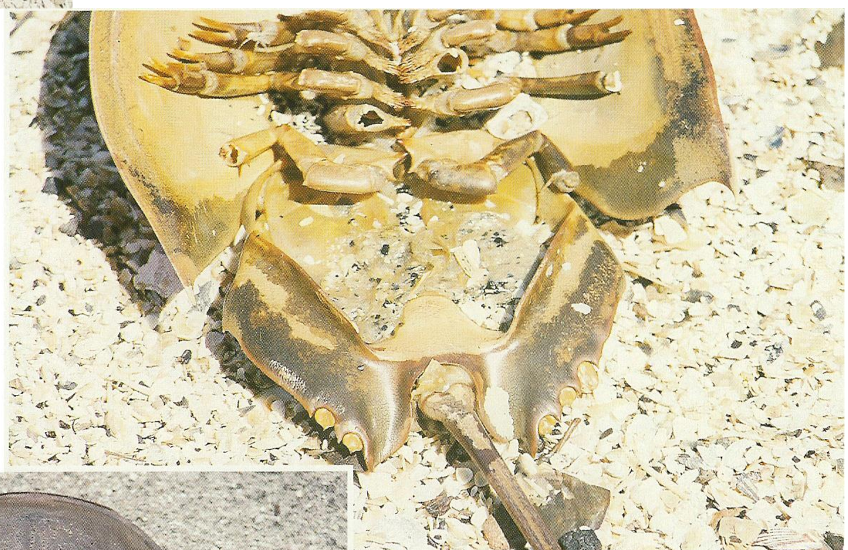
In 1958, however, Drs. Waterman and Ripley from Yale University and the Yale Peabody Museum, searched in vain for specimens of *T. hoeveni* not only in museum collections all over the world, but also in the Moluccas. The fishermen on Halmahera who are well acquainted with the marine fauna around Ternate, Tidore and the other small outlying islands insist that the nearest place where horseshoe crabs are caught regularly is in Manado and along the Sulawesi coastline 200 miles towards west. Dr. Ripley spent three months in that area without finding even a trace of horseshoe crabs, and shortly afterwards Dr. Talbot Waterman in an excellent article concluded that *T. hoeveni* is in fact a somewhat abnormal specimen of *Tachypleus gigas.*

Limulus virescens, too, has faded into oblivion.

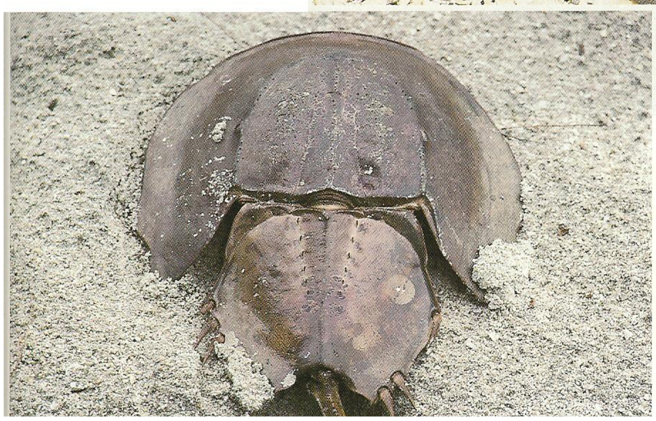
But one question of great interest remains: Is it still possible to find *T. gigas* or any other horseshoe crab species alive on the Moluccas or Sulawesi?

Among scientific workers (but seldom among fishermen) you may sometimes encounter the belief that only one species of horseshoe crab *(*King-Crab = *Limulus moluccanus* = *Tachypleus gigas)* is found along the Southeast Asian shores. It is therefore of interest to note that laymen in several countries recognize the distinction between the three Indo-Pacific species.

Abnormal development (following injury?) of the lateral, movable spines on both sides of the opisthosoma of a female of *T. gigas.*



Opisthosomal deformity, ventral view.



Abnormal development (following injury?) of lateral, movable spines in a male *T. gigas.*

(Port Dickson, Malaysia)

Dorsal view of four adult horseshoe crabs. From left to right: A female *C. rotundicauda* (China), a male *T. gigas* (Moluccas), a male *C. rotundicauda* (Port Dickson, Malaysia) and a female *C. rotundicauda* (China).

Ventral view of the same four horseshoe crab specimens.

In Singapore the different names used for the two existing species are: Belangkas *(T. gigas)* and keroncho *(Carcinoscorpius rotundicauda).* The term "belangkas" is also in use in the Malay Peninsula, where, however, the term "belangkas padi" replaces "keroncho" for *C. rotundicauda,* the smaller species. The name "belangkas" is given as "king-crab", "keroncho" means the young. Apparently the smaller size of *C. rotundicauda* is the cause of the use of this name. The eggs, "telor belangkas" are much sought after as a delicacy, especially by pregnant women, and it is said that the king-crab is regarded by strict Mohammedans as "haram", but that liking sometimes discounts the religious ban. I have found dozens of burnt females especially in the southeastern part of Malaysia, and I was told by the local population that live females of *T. gigas* are often caught during spawning and thrown ventral side up into a fire where the heat will crack open the prosoma along the exuviation suture and allow the dinner guests to dig out the eggs.

In India, mating couples of *T. gigas* are locally known as *Ram-Lakhan magar.* The allusion is to the two brothers, the elder Rama and the younger Laksmana, who are heroes of the ancient Hindu epic, Ramayana. Magar in Hindi means a crocodile, the bigger one being Ram and the smaller male Lakhan.

The two species in Thai waters are distinguished by the names *mangda* (the species with the smooth caudal spine, or *C. rotundicauda),* and *hela* or *hera* (the species with the dorsally serrate spine, or *T. gigas).*

Mangda is used for food, its egg mass is eaten cooked, often served in a curry. Hela, on the other hand, is considered toxic and never eaten in Thailand, although the eggs, as mentioned above, are a delicacy in Malaysia.

Several articles in medical literature have reported on intoxications following the ingestion of eggs or meat from *T. gigas and C. rotundicauda,* and the American Dr. Halstead has identified two strong alkaloids in the eggs and tissue of these two species. As far as I am informed, no one has died from such intoxications yet, but the alkaloids are potentially strong enough to cause death in human beings...

A burnt prosoma of a female *T. gigas* found together with numerous others in the same condition, on the beach north of Desaru, Johor, Malaysia.

The female *T. gigas* carrying the male on her back is scooping a hole in the sand for the deposition and fertilization of part of her egg mass. (Tonggok, north of Kuantan, Malaysia)

A female *T. gigas* has died during the deposition of the eggs and nest has been robbed by unknown predators. (Kampong Cherating, Malaysia)

The horseshoe crabs are also greatly appreciated on the dinner tables in Borneo, Viet Nam and the Philippines. In China, *T. gigas* is known as the *tiger horseshoe crab, C. rotundicauda* as the *devil horseshoe crab* and the biggest species of them all, *T. tridentatus,* is generally known as either the *three spine horseshoe crab,* or, the *eastern horseshoe crab fish.*

As suggested above, *T. gigas* occurs discontinually along the Southeast Asian coastlines, just as is the case for the two other Indo-Pacific species and also the American *Limulus.* And just as it may be difficult to find sizable populations of adult animals, it may certainly also be difficult to locate the nests. In 1906, Iwanoff observed that in Java, in the Kingdoms of Batavia, Karwang and Bantam, with a total coastline of some 350 km, the only place where eggs were laid was near the fishing village of Sadari. Likewise, one of the very few places in India where eggs have been found is the Burhabalang river near Chandipore.

Previous authors never even had to look for nests : "On the coast of Bengal *L. moluccanus (T. gigas)* breeds at the end of the cold season, i.e. in March. The eggs, which are not very numerous, have a green colour and measure about 3 mm in diameter, are carried on the ventral surface of the abdominal appendages, to which they adhere lightly". (Annandale, 1909). Similar misconceptions have been published by other workers, e.g. Smedley, in 1929: ". . . as neither this species *(T. gigas)* nor *rotundicauda* buries its eggs as do the American and Chinese forms, but carries them about attached to the swimmerets."

And even earlier, in 1883, Willemoes-Suhm on the testimony of local inhabitants in the Philippines and East Indies, stated that the eggs of both *C. rotundicauda* and *T. gigas* are not laid in the sand but are carried by the female on its abdominal appendages.

Today we know that the female T *gigas* produces approximately 8.000 eggs per year, with a mean diameter of 1.75 mm when newly laid. She deposits her eggs from March to June in nests each containing approximately 400 eggs. The number of days from fertilization to hatching is 32-35 and the babies will moult 2-3 times during the year of their hatching. The ratio of males to females on breeding beaches is an orderly 1:1.

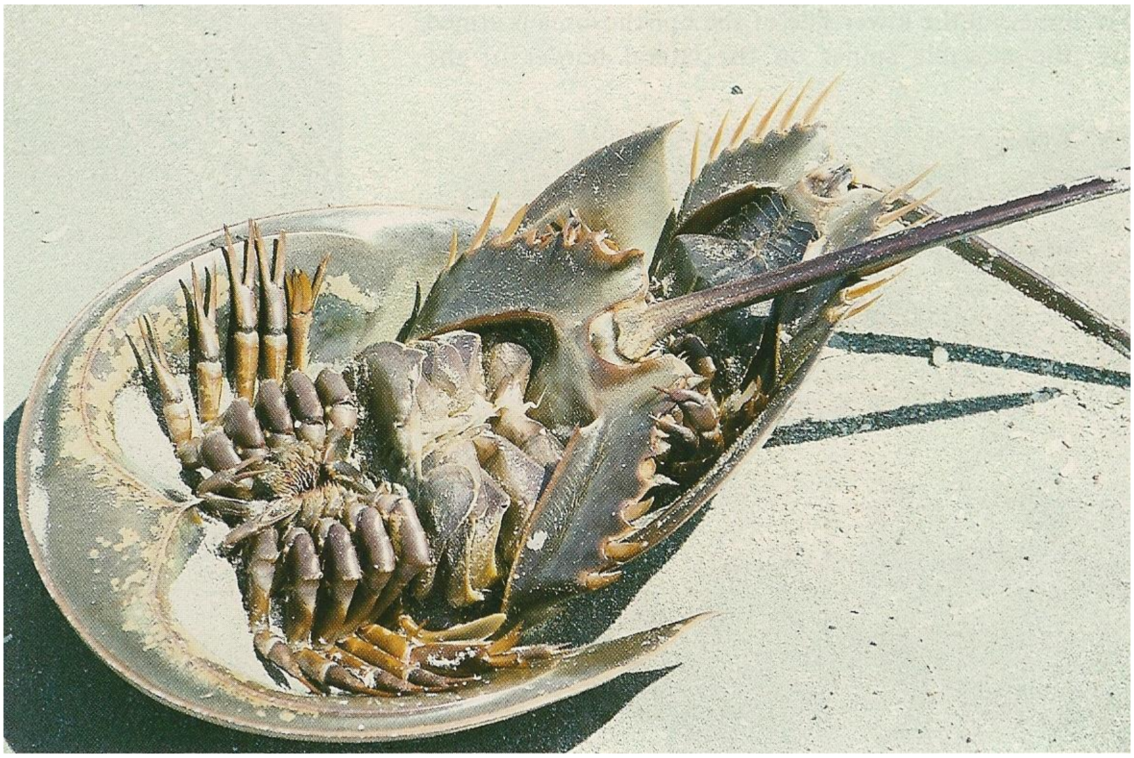
The pale olive-green eggs are laid in mud or sand in the intertidal zone of protected beaches or in the muddy or swampy mouth of a river or a creek, subject to tidal influences and thus quite often alternatively covered by brackish water and exposed to challenging weather conditions. The eggs are buried approximately 15 cm into the substratum.

A mating couple of T. *gigas* encountered in the river estuary near the city of Mersing, Malaysia.

A mating couple of *T. gigas* turned over on the beach near Kampong Pengerang, Johor, Malaysia.

The following observations, made on March 4 and 5, 1939, by Drs. B. N. Chopra and H. S. Rao at the Chandipore beach have been borrowed from the records of one of the Field Station Books of the Zoological Survey of India. The animals were observed on the open intertidal beach which consists of firm mud and sand:

"A few specimens of *Limulus (=T. gigas)* buried in sand (nearly always in pairs), some of them carrying sea-anemones and barnacles were found near the fishermen's traps. The pairs always consisted of the smaller one riding on the back of the larger (probably the female), holding on to the abdominal part of the larger specimen by its first two pairs of legs to the last two pairs of the lateral spines of the abdomen.



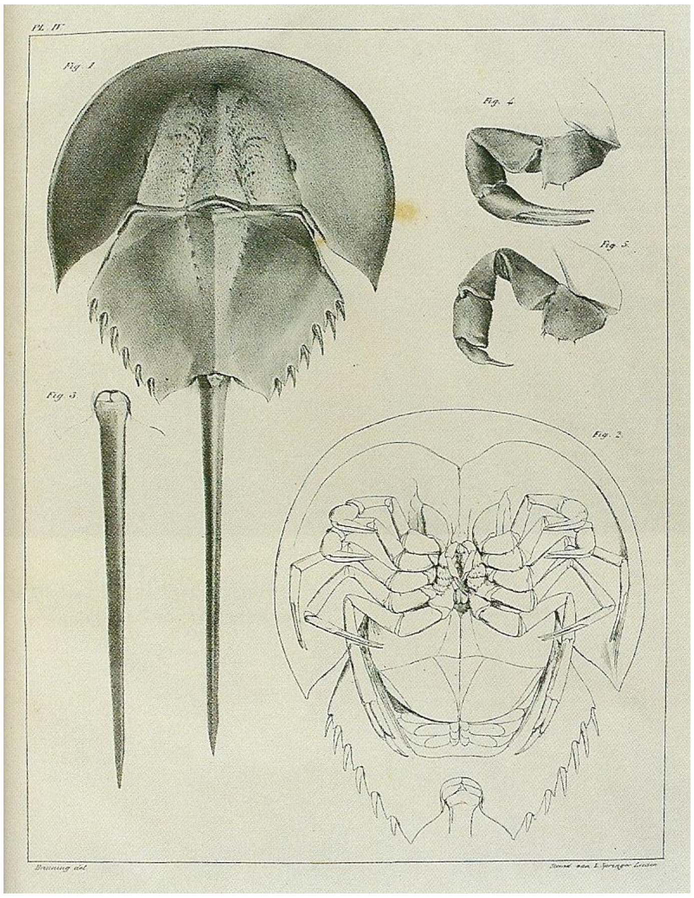
The small male is striving to turn the couple of *T. gigas* back again by means of its hard, stiff telson.

Notice the two pairs of hemichelate claspers of the male *T. gigas* holding on firmly to the opisthosoma of the female

There seems to be some variation in the manner of the smaller individual attaching itself to the larger and also in the reduction of the abdominal spines, Nos. 4 to 6. The 4th and 5th spines of the abdomen of the larger specimen are reduced. All the six spines of the smaller specimen are normally long. The actinians and barnacles are found usually on the smaller specimens, even covering the eyes in some cases. When the two are separated forcibly and placed on dry land the larger one moves down to the water while the smaller appears to be helpless and does not usually follow its mate. Placed close together, however, the smaller gets on to its normal position over the back of the larger."

Females of *T. gigas* may exceed half a meter in total length and (as usual) the caudal spine or telson equals approximately half the total length of the body. Special features distinguishing *T. gigas* include a movable spur present at the apex 'of the fourth segment of the sixth prosomatic appendage. A postanal spine which is triangular in section, crested on top with a hollow underside. The second and third pairs of prosomatic appendages are modified as hemichelate claspers in the male, with the immovable finger stumpy and abbreviated while the movable one is thick in the middle, slender and cylindrical distally. The lateral crest bordering the gill-chamber on the lower side of the opisthosoma is armed with a distinct spike, and, finally, the movable spines on the lateral border of the opisthosoma are very dissimilar in the two sexes, of equal length and very long in the male *and young female,* the posterior three short and apically acute in the adult female.

Sunset over the Strait of Malacca seen from the Port Dickson.



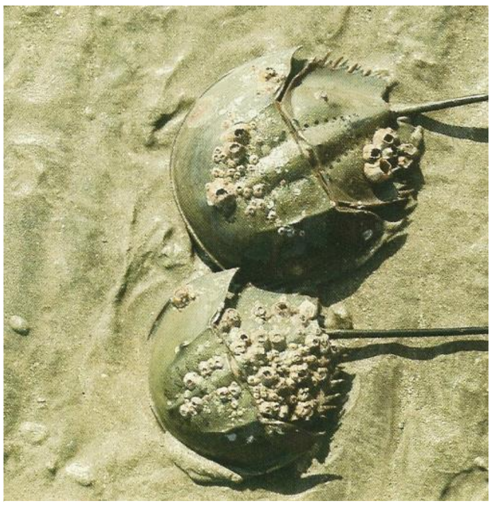
Limulus (Carcinoscorpius) rotundicauda presented in a manuscript drawing from 1838. Fig. 1 depicts the female, and Fig. 2 the male of this species. Fig. 4 and Fig. 5 show anatomical detail from *Limulus longispina (T. tridentatus)* for comparison. (J. van der Hoeven)

Although the biogeographical range of *C. rotundicauda* is as extensive as the ranges of the two other Oriental horseshoe crab species, its preferred habitat in muddy rivers, swampy estuaries and inaccessible mangroves make it extremely difficult to explore its lifecycle in any detail. *C. rotundicauda,* still locally (and very aptly) known as the *Estuarine King-Crab,* occurs in the extreme northern part of the Bay of Bengal where it has been caught at Calcutta, up the Hoogly River, about 90 miles from the open sea.

It lives along the west coast of Malaysia (including Pinang Island) in the estuary of Langat River, around Tanjong Tuan and in the estuaries of Linggi and Muar rivers. You may find it in Singapore and, discontinually, along the east coast of the Malay Peninsula all the way up north to Kota Bharu and the Kelantan River mouth.

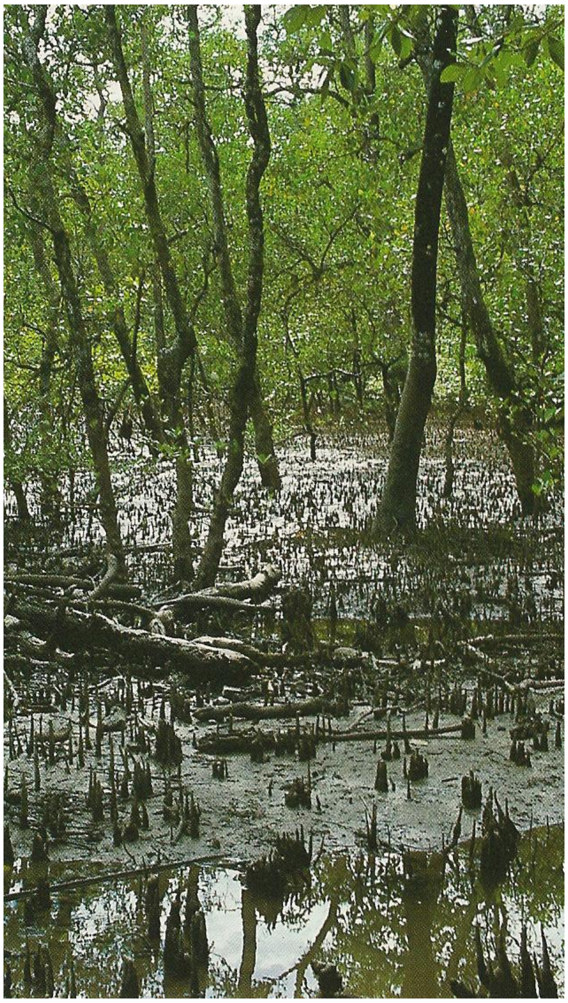
In Thailand it has been found in a river about 4 kilometres from the sea, and it can be caught in Chon Buri about 50 kilometres southeast of Samut Prakan and at the mouth of Mae Nam River in this area. Specimens of *C. rotundicauda* believed to have been responsible for incidents of food poisoning have been bought in Samut Sakhon, a town situated on the northern coast of the Gulf of Siam, an area well known for the breeding of this species.

One specimen has been recorded from Zamboanga on the westernmost tip of Mindanao in the Philippines, and earlier reports have mentioned its presence in Indonesia and the Moluccas.



Dorsal and Ventral view of female (larger) and male of the species *C. rotundicauda.* Note the heavy growth of various organisms on the prosoma and opisthosoma of both specimens.

(Bako, Sarawak, Borneo)



Where tropical rainforest and mangrove swamp meet.
(Bako, Sarawak, Borneo)

In Borneo (Kalimantan) *C. rotundicauda* is frequently found in the estuarine areas of Datu Bay, and a particularly good location to study this species is the huge estuary between Bako and Santubong Island, which is subject to strong tidal influences. But since the water is seldom clear in this area, it may be a matter of great patience to obtain significant results. As mentioned above, the biggest specimen of *C. rotundicauda* I have seen was found dying in the mangrove swamp surrounding Bako after a violent thunderstorm at new moon.
C. rotundicauda exists discontinually along the northwestern coast of Sarawak as far as Miri, and it has also been reported to live in Sabah and Brunei. Viet Nam has a population of this species in the northern part of the country where it overlaps with *T. gigas* and *T. tridentatus.*
The only place where this author has seen an abundance of *C. rotundicauda* is on the vast intertidal mud flats west of Beihai city, in the northernmost part of the Tonkin Gulf, known as Beibu wan (Beibu Gulf), on the beautiful shores of Zhuang Autonomous Region of Guangxi, China. This species is known locally as "devil horseshoe crab" or "ghost horseshoe crab". On June 7th, 1985, my experienced Chinese colleagues together with local fishermen succeeded in collecting 218 specimens in the course of a few hours. The collection was made during low tide with the mud flats almost fully exposed and nearly all the animals were found in the back country completely buried in the heavy mud.

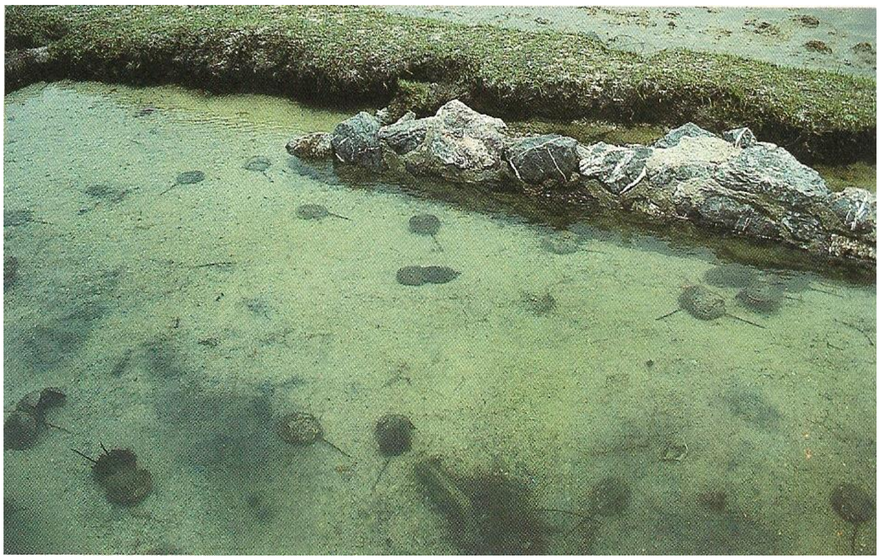
C. rotundicauda lives on Weizhou Island, on the southern coastline of Hainan Island (in the vicinity of Yulin) and, in fairly abundant numbers, east of Leizhou Peninsula.

Hunting horseshoe crabs and nests in the mangrove swamps on Borneo does indeed require a sensible choice of footwear.

Clockwise from top: A female *T. gigas*, a male *T. gigas* and a female of the species *C. rotundicauda.* Note the obvious and species specific differences in the external anatomy of the two species.

(Bako, Sarawak, Borneo)

Adult male of *Carcinoscorpius rotundicauda.*
(Bako, Sarawak, Borneo)



A natural brackish water pond containing 100 pairs of *C. rotundicauda* collected for studying and partial bleeding.

(Beihai, China)

I have never seen *C. rotundicauda* east of Macao, and have never heard or read about its presence in Hong Kong, Taiwan Island or further east or north. But this species is certainly discreet, even secretive during most of its lifecycle, and it may probably easily have been overlooked in these and other areas.

In Thailand, the breeding season has been reported to be continuous, while in Borneo and China, at least, the breeding season lasts from March/April to June/July. The female carries about 10.000 eggs with a diameter of approximately 2.0 mm and she deposits her eggs in clusters of 80-150 eggs per nest. The nests are located in the mud of a sea-shore or a river bed, and they are only about 2 to 5 cm deep. The eggs will hatch in the course of 32 to 35 days and the hatchlings will moult 2 to 3 times during their first year of life.

C. rotundicauda is considerably smaller than the other three extant species of horseshoe crabs and their carapace is more rounded, quite often resembling a saucer. This species has no spur on the apex of the fourth segment of the last appendage of the prosoma. The postanal spine is cylindrical, neither crested on top nor with a grooved underside. The second and third pairs of prosomal appendages in the male are modified into completely chelate claspers with long, slender fingers, similar in form.

The lateral, moveable spines bordering the opisthosoma are comparatively short in both sexes, decreasing in length from front to back. The second and third moveable spines tend to be the longest.

During much of its lifecycle C *rotundicauda* appears to be sympatric with either *T. gigas, T. tridentatus,* or both species, on a local as well as a regional scale.

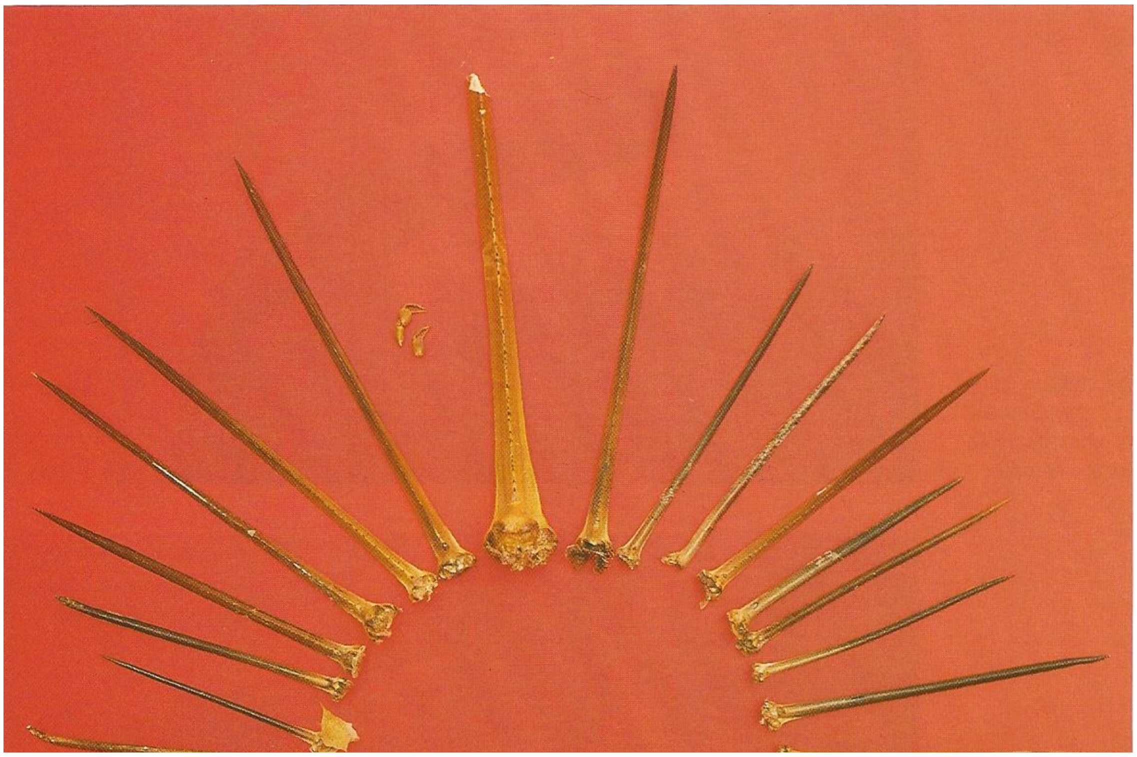
Close up ventral view of a mating pair of *C. rotundicauda. N*ote the completely chelate claspers of the male.

(Beihai, China)

Ventral view of a juvenile *Carcinscorpius* found buried in the intertidal zone. The colour of the carapace is completely black.

(Bako, Sarawak, Borneo)

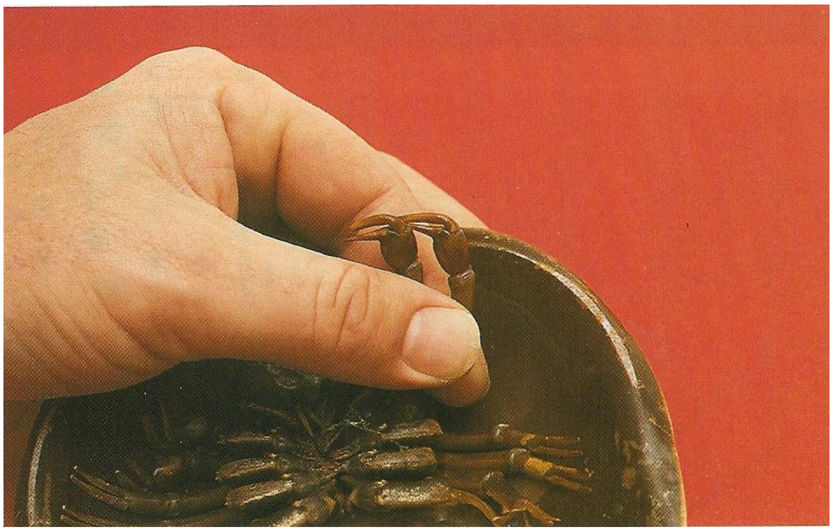
A male *T. gigas,* left, and a female *C. rotundicauda* on the beach at Bako, Sarawak. This is the biggest specimen of *C. rotundicauda* I have seen. Prosomal width: 16.1 cm. Total length: 34.8 cm. Length of telson alone: 18.4 cm. The specimen has been preserved.



A collection of telsons from *C. rotundicauda* (generally smooth and cylindrical, no grooved underside), *T. gigas* (generally the postanal spine is triangular, crested on top with a hollow underside) and *T. tridentatus* (centre, broken).
To the left of the broken telson from *T. tridentatus* you see the chelate claspers from a male *C. rotundicauda. All* telsons are collected from dead specimens found in Borneo, the Philippines, Malaysia, the area of Hong Kong *(T. tridentatus)* and China.

Ventral view of an adult male *C. rotundicauda,*

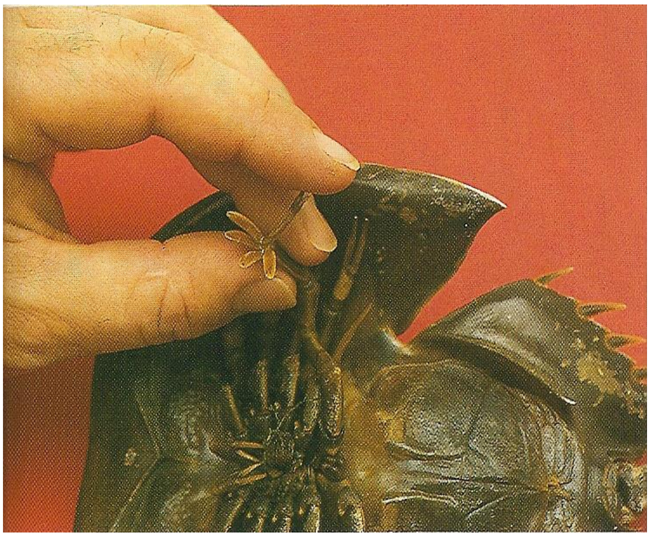
(Beihai, China)



The completely chelate clappers of the male *C. rotundicauda* with which it attaches itself to the opisthosoma of the female.

Gill books and ventral view of a male *C. rotundicauda.* (Beihai, China)

Based upon field observations in southern China, where you may find all three Oriental species living virtually side by side, it is my *experience* that *T. tridentatus* and *C. rotundicauda* spawn in the exact same area, and it is my *impression that you may find areas in southern Chinese waters where all three species spawn in perfect sympatry.*



Sixth pair of appendages of *C. rotundicauda* with conical and somewhat pointed sclerites preventing the animal from getting stuck in the soft mud of its natural habitat.

A fully mature *C. rotundicauda* meets two, by comparison, gigantic adults of the species *Tachypleus tridentatus*

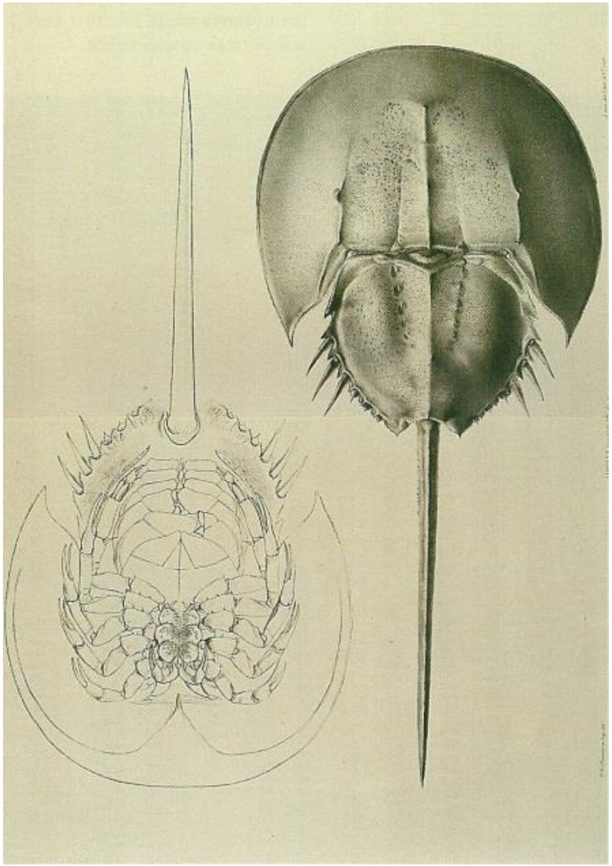
A busy day is turning into a busy night in Bako, Sarawak, Borneo.

Tachypleus tridentatus

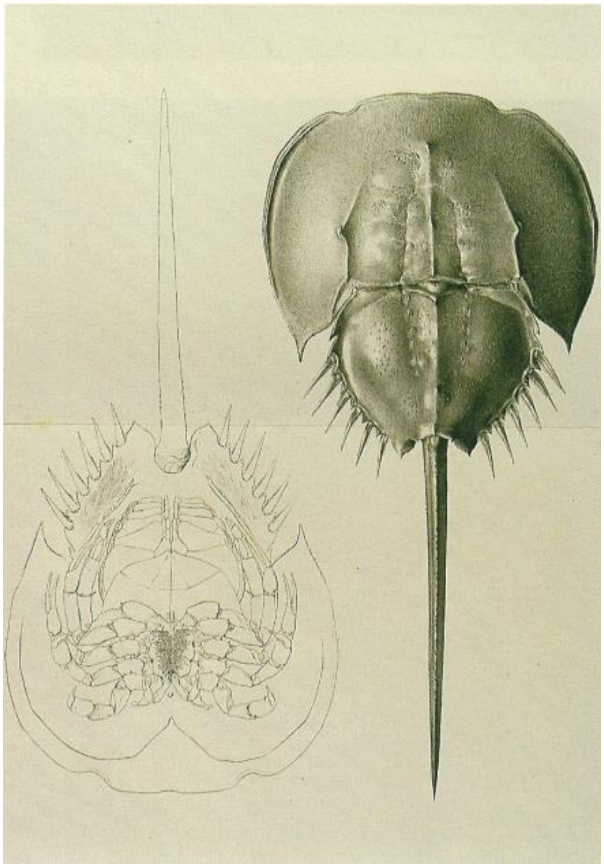
The Japanese *T. tridentatus* occurs in the western half of the Inland Sea and off the northern coast of Kyushu as far west as the Prefecture of Fukuoka. One of the best places to observe its spawning habits and collect embryological material is supposed to be the coasts of Okayama Prefecture on the Inland Sea.

Observations have been conducted by Japanese scientists at the villages of Yosuna and Oehama near the town of Kasaoka close to the border separating Okayama and Hiroshima Prefectures, where there are both sandy beaches and extensive mud flats close together, a condition which appears to be necessary for the spawning and growth of the Japanese horseshoe crab.

In Japan, due to overfishing, pollution and land reclamation, the horseshoe crab was almost obliterated, but, fortunately, the Japanese government took the advice of the scientific community and today regulates the taking of *Kabutogani* or *Umi-do-game* as the Japanese species - now considered as a national treasure - is called.



The female of *Limulus longi-spine (Tachypleus tridentatus)* from Ph. Fr. de Siebold "Fauna Japonica", 1850.



A masterpiece from 1850 depicting a male *Limulus longispina (Tachypleus tridentatus)* in the accompanying text also named *Kabuto-gani* and *Cancer loricatus.*

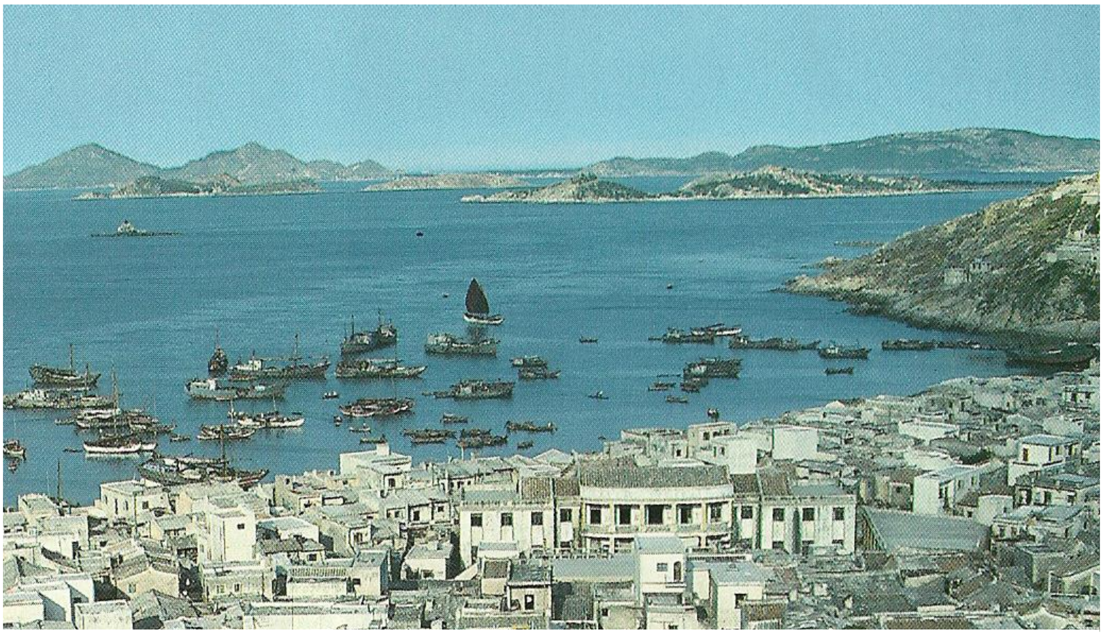
A mating couple of *T. tridentatus.*

A close-up look of a mating pair of T. *tridentatus* on the beach just outside the Department of Oceanography, Xiamen University, China.

Apart from a specimen recently reported from Sumatra it seems that *T. tridentatus* ranges much further north than the other two Indo-Pacific species. It has been found on the northern coast of Borneo (the British Museum has a specimen from Kudat) and on the western islands of the Philippines, but major concentrations are to be found along the Vietnamese coastline at least as far south as Nha-trang, on Hainan Island and in Hong Kong, along the South China coast, and on Taiwan Island, and along the east coast of China at least as far north as Hangzhou and Shanghai.

In Japan and in some areas of China, *T. tridentatus* seems to be the only representative of the three Oriental species, while as far north as Hong Kong you may easily find *T. tridentatus* and *T. gigas* (even overlapping each other) in such areas as Deep Bay, Tai Po (To-lo Harbour) and on the east coast of Pengchau Island in Mirs Bay. In Hong Kong you should ask for an "underwater monkey" if you want a horseshoe crab.

Until recently we have had very limited information about the Chinese horseshoe crabs in international scientific literature. Sporadic articles have been accessible in the Chinese language (sometimes with English summaries), but these works discuss, primarily, the morphology and physiology of the blood cells (amebocytes) of the animals rather than more common subjects such as their biogeographical range and general ecology.



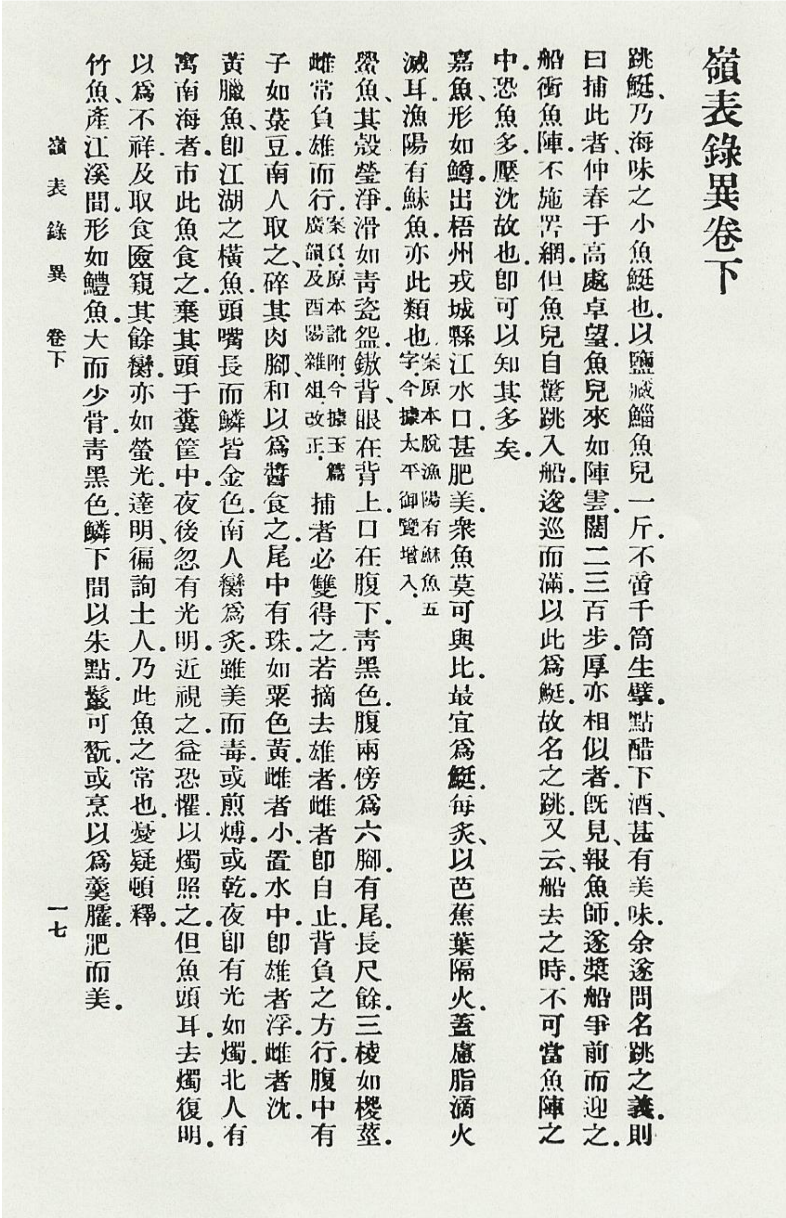
The South China Sea seen from Dongshan Island, Fujian, China. *T. tridentatus* abounds in these waters where no other horseshoe crab species exists.

Professor Fang Yongqiang from the Third Institute of Oceanography, National Bureau of Oceanography, in Xiamen, presenting two beautiful females of *T. tridentatus.*

Friends and colleagues in Beihai. In the middle Mr. Zhou, an engineer, holding possibly the largest specimen of *T. tridentatus* recorded so far: The female weighed 7.9kg when caught.

After a long and fruitful day studying and collecting horseshoe crabs, Mr. Liang Guangyao and Mr. Zhang Guihang head back through the muddy channels, breeding ground for *C. rotundicauda* and *T. tridentatus.* (Beihai, China)

To my knowledge, nothing has as yet been published on these subjects outside of China, which is the reason why I take this opportunity to communicate the information I have been able to collect during visits to Shanghai, Xiamen (Amoy) and, especially, to Beihai, Zhuang Autonomous Region of Guangxi where I have had several opportunities to study the various species in some detail in their natural habitat expertly assisted by a number of Chinese scientists.



嶺表錄異卷下

跳鯅、乃海味之小魚鯅也．以鹽藏鰡魚兒一斤．不啻千筒生擘．點醋下酒、甚有美味．余遂問名跳之義．則曰捕此者、仲春于高處卓望魚兒來如陣雲．闊二三百步．厚亦相似者．既見、報魚師．遂槳船爭前而迎之．船衝魚陣．不施罟網．但魚兒自驚跳入船．逡巡而滿．以此爲鯅．故名之跳．又云、船去之時．不可當魚陣之中．恐魚多．壓沈故也．卽可以知其多矣．

嘉魚、形如鱒．出梧州戎城縣江水口．甚肥美．衆魚莫可與比．最宜爲鯅．每炙、以芭蕉葉隔火．蓋慮脂滴火滅耳．漁陽有鯠魚．亦此類也．案原本脫漁陽有鯠魚五字．今據太平御覽增入．

鱟魚、其殼瑩淨．滑如青瓷盌．鏉背、眼在背上．口在腹下．青黑色．腹兩傍爲六腳．有尾．長尺餘．三棱如椶莖．雌常負雄而行．案負．原本訛附．今據玉篇廣韻及酉陽雜俎改正．捕者必雙得之．若摘去雄者．雌者卽自止．背負之方行．腹中有子如菉豆．南人取之、碎其肉腳、和以爲醬食之．尾中有珠．如粟色黃．雌者小．置水中．卽雄者浮．雌者沈．

黃臘魚、卽江湖之橫魚．頭嘴長而鱗皆金色．南人臠爲炙．雖美而毒．或煎煿．或乾．夜卽有光如燭．北人有寓南海者．市此魚食之．棄其頭于糞筐中．夜後忽有光明．近視之．益恐懼．以燭照之．但魚頭耳．去燭復明．以爲不祥．及取食匳寬其餘臠．亦如螢光．達明、偏詢土人．乃此魚之常也．憂疑頓釋．

竹魚、產江溪間．形如鱧魚．大而少骨．青黑色．鱗下間以朱點．鮮可翫．或烹以爲羹臛．肥而美．

嶺表錄異　卷下　一七

Ling – biao – lu – yi.

Maybe the earliest source of written information on the horseshoe crabs is the work *Shan-hai-jin* (Classic of the Mountains and Rivers) (editor unknown) which was published before the Western Han Dynasty (206 BC-AD 24). One edition includes a description of the size, shape, behaviour and use of the horseshoe crab written by a Chinese scholar named Kuo Po.

During the Tang Dynasty (AD 618-907) two works mentioning the living fossils appeared: *Ben-cao-shi-yi* (Omissions from Previous Pharmacopoeia) published by Zhen Cangqia who says that the horseshoe crabs "live in the south sea, both big and small ones are in pairs of opposite sex".

The meat is described as "peppery, salty and slightly poisonous", and *Ling-nan-shi-yi* (Omissions from Record of South of the Passes) written by Liu Xun "The male is small and the female big. The male floats in the water and the female sinks".

All these three books are extremely rare in China. With the kind help of Dr. Tao Zong-jim, Shanghai, I succeeded in getting permission to reproduce a few pages from two other fascinating books.

One is the famous *Ben-cao-gang-mu* (Compendium of Materia Medica) written by Li Shizhen (1518-1593) during the Ming Dynasty (1368-1644) and published in Chinese in 1596. Already in 1606 *Ben-cao-gang-mu* was translated into Japanese, and it has later been translated into Latin, French, German, English and Russian.

The other work to be presented here is *Ling-biao-lu-yi,* but unfortunately I do not know the name of the author or the publishing year.

All these books were written in ancient Chinese and it is not easy to translate them into English. However, I hope the following excerpts may give at least an impression of how the horseshoe crabs were described by scholars in ancient China.

Ling-biao-lu-yi:

Its shell is lustrous and clean, and as smooth as a blue porcelain dish. The shape of its back is like "Ao" (a kind of cauldron used in ancient China). Its eyes are on the back. The mouth is at the lower part of the abdomen. It has a tail which is more than one chi ("chi" is a unit of length. The absolute length of "chi" varies in different historical periods. Today, one chi equals 1/3 meter), triangular in shape like the stem of a palm. The male horseshoe crab often loads on the female while moving. They are always fished in couples. If the male horseshoe crab is taken away, the female stops moving immediately.

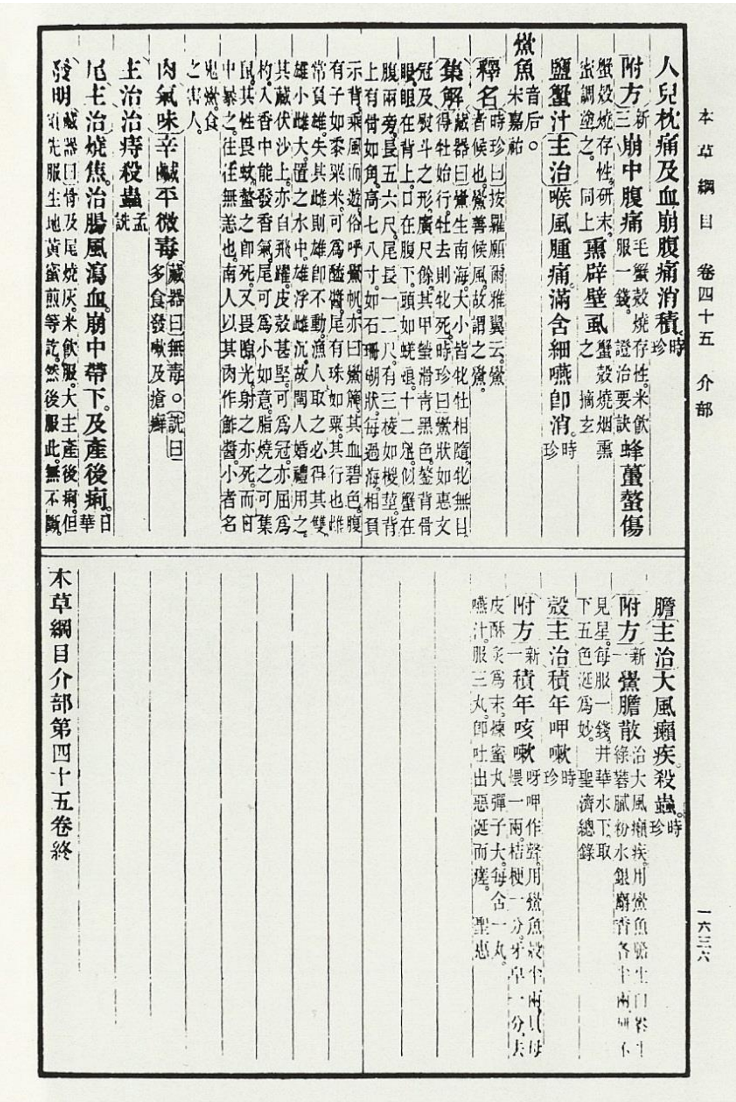
A Chinese village impression by a Danish artist.

There are eggs in the abdomen which are like mung beans. People in the southern part of China blend the meat of the claws with sauce for food. There is a pearl in the tail which is like millet and yellow in color. The pearl in the female horseshoe crab is smaller. If it is laid on water, the pearl in male floats and in female it sinks".

Ben-cao-gang-mu:

"The horseshoe crab is like a Hui-Wen hat (a kind of hat used in ancient China) or iron, and has a width of more than one chi. Its shell is lustrous, and smooth, blue-black in color. Its back is like "Ao". The eyes are on the back within the bone, and the mouth is at the lower part of abdomen.

The head is like a dung beetle. It has 12 claws like a crab, located on both sides of the abdomen, which are 5-6 chi long. The tail is 1-2 chi long, triangular in shape. There is a spine, like a horn, on the back which is 7-8 can (1 can equals 1/10 of a chi) in height like Shi-Shan-Hu (this expression is unknown, but it could mean a kind of coral). When crossing the sea, the male loads on the back of the female. The couple rides the wind swimming. So the local name for the horseshoe crab is horse-shoe crab sail or horseshoe crab raft. Its blood is blue.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"Ben-cao-gang-mu" by Li Shi zhen, 1596.

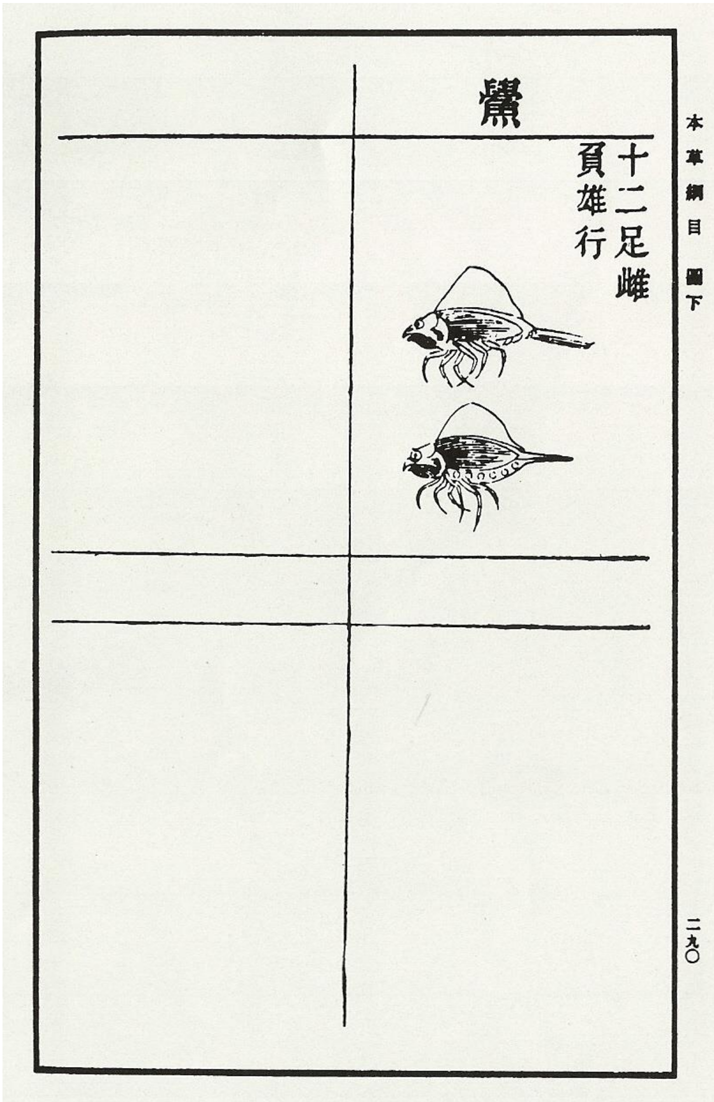


Illustration from Ben-cao-gang-mu. The legend on top of the illustration reads: "horseshoe crab". In the upper right-hand corner, it reads: "12 feet. Male loads on top of female when moving".

There are eggs in the belly which are the size of millet. They can be used to make sauce. In the tail there is a pearl like millet. While moving the male horseshoe crab often loads on the female. If the female horseshoe crab is taken away, the male stops moving. The fisherman always gets it in couples.

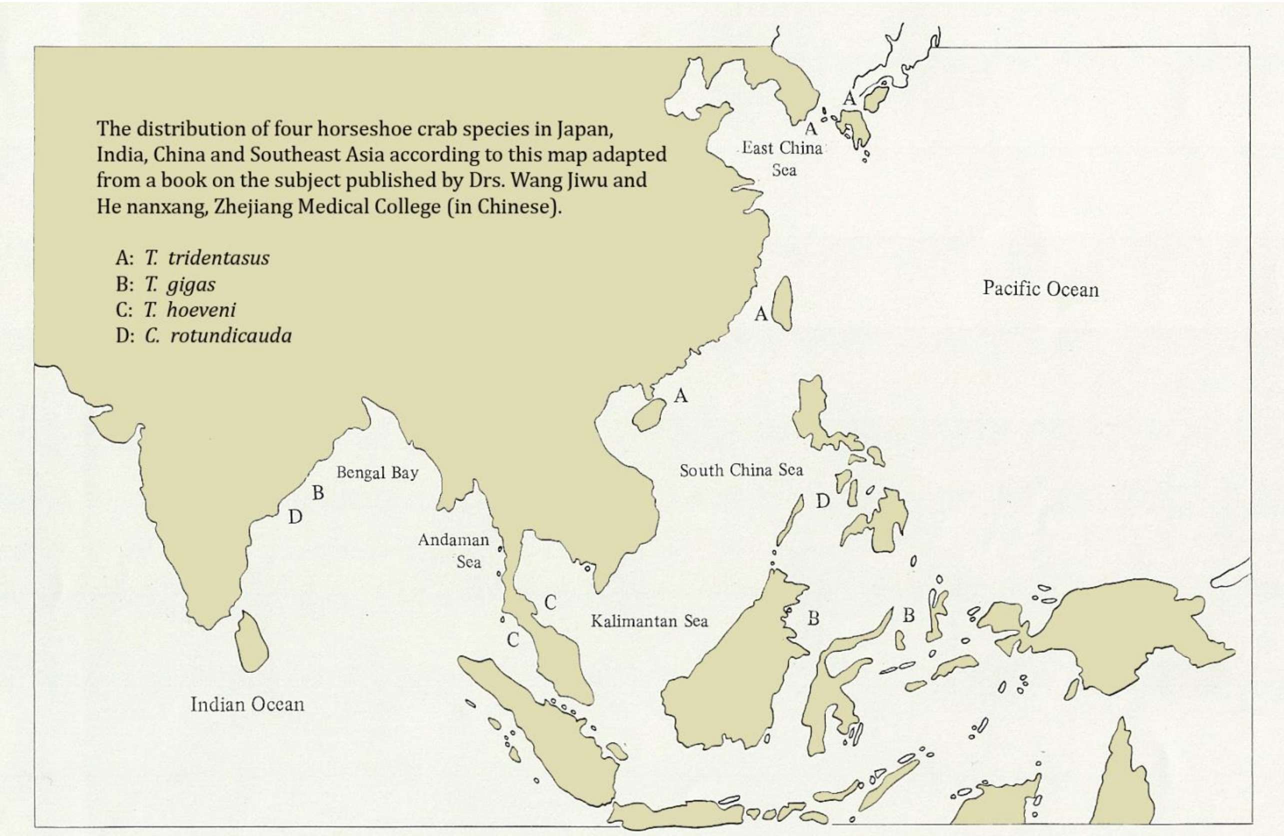
The male is smaller than the female. When it is placed in the water, the male floats and the female sinks. Therefore the people in "Min" (Fujian Province and its vicinity) use it for wedding celebrations. The horseshoe crab hides itself in the sand, sometimes it jumps up. The shell is quite stable and can be used as hat or ladle. When it is mixed with perfume, it stimulates the intensity of the fragrance. The tail can be used to make small "Ruyi" (an S-shaped ornamental object, usually made of jade, a symbol of good luck). Burning the lipid attracts rats. The horseshoe crab fears the mosquitos. It dies if it is bitten by mosquitos. It fears spot light, too, and dies after exposure to concentrated light. It is, however, not scared to be exposed to sunlight, not even in the mid-day. The southern people use its meat to make salted fish sauce. A smaller size species of horseshoe crab is called ghost-horseshoe crab. It is harmful to humans if they eat it."

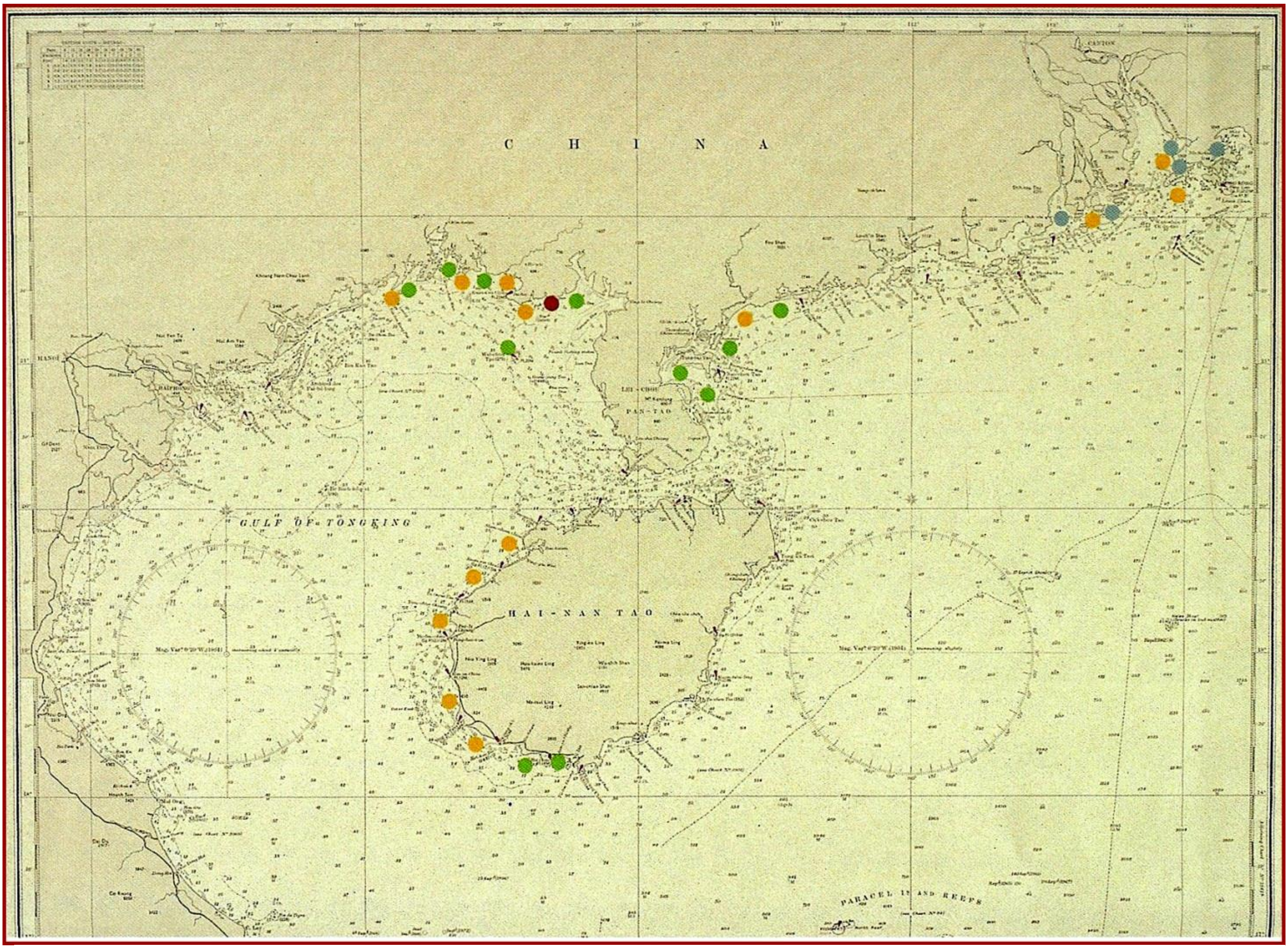
According to ancient Chinese literature, one or two species of horseshoe crabs were known to occur in eastern and southern China.

In the last couple of years, my Chinese colleagues and I have succeeded in identifying all three known Oriental species along a relatively short stretch of the Beibu Gulf coastline, east and west of Beihai City.

And, additionally, I have in my collection of preserved Chinese specimens and exuviae eight females which I found dead on Pak-fu-tau beach, east of Beihai City. All the eight specimens were found within the same small area in the month of June, apparently washed upon the beach by the tide.

Unfortunately, all the eight females have badly damaged ventral surfaces including completely collapsed and dried out gill books, but the general impression is that all specimens are elderly, sexually mature animals and thus too small for either *T. tridentatus* or *T gigas.* On the edges of the opisthosoma all the eight have lateral, movable spines of equal length instead of the three abbreviated posterior spines one would traditionally expect to find in adult females of *Tachypleus*.





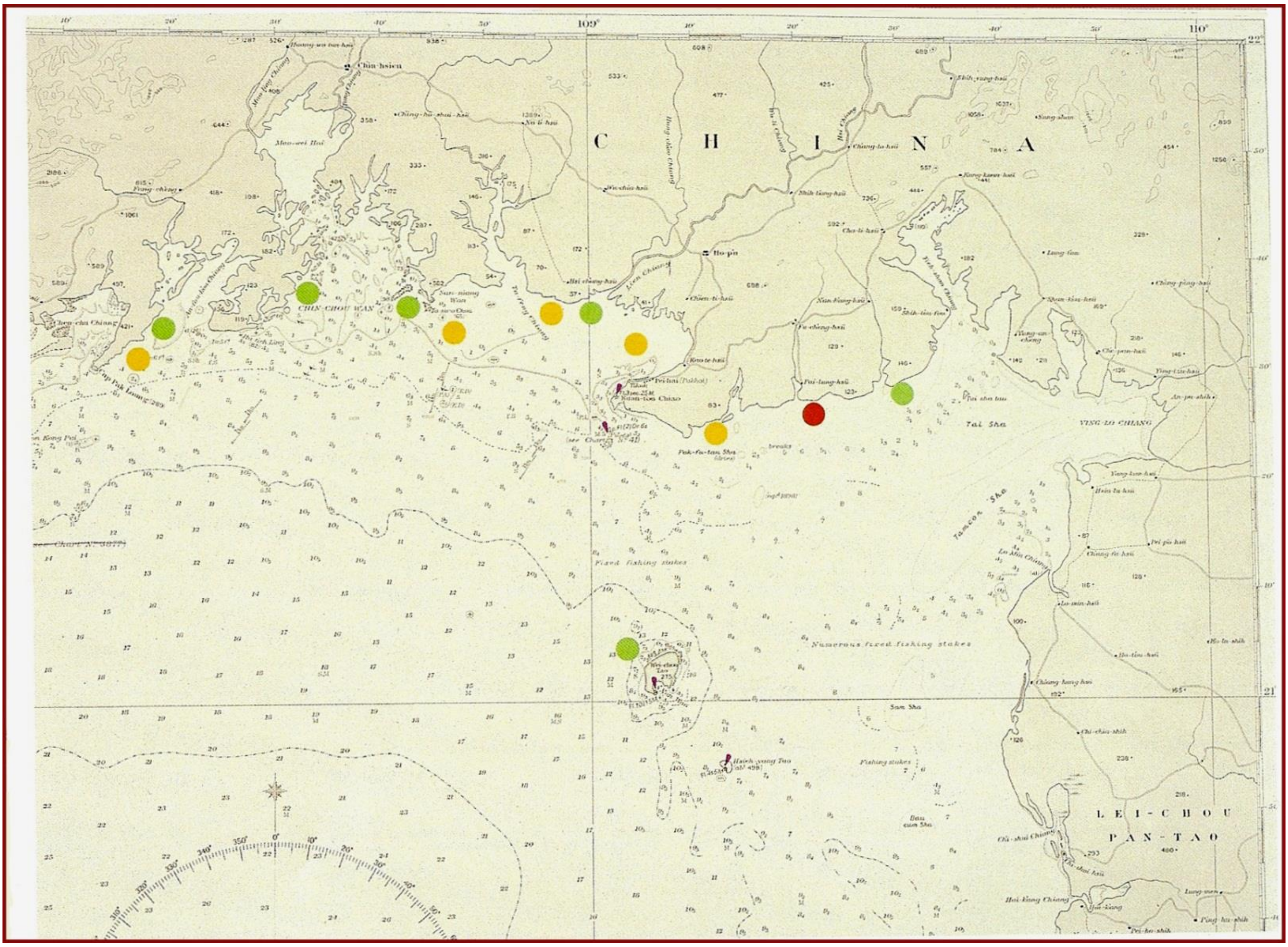
The distribution of the Chinese horseshoe crab species according to the joint Chinese-Danish surveys carried out in 1984 and 1985.

T. Gigas

T. tridentatus

C. rotundicauda

A new (sub-)species?



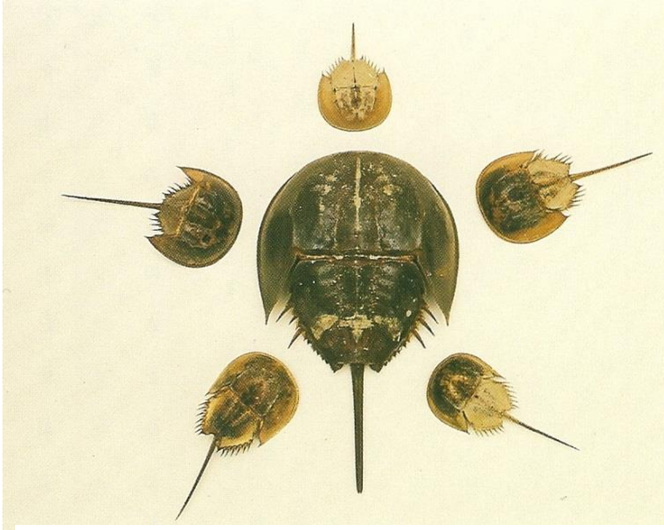
More detailed map of the distribution of horseshoe crab species in Beibu Gulf

C. rotundicauda

T. tridentatus

A new (sub-)species?

Outside Guangxi Institute of Oceanography, my friends and ardent helpers (L. to R.): Mr. Liang Guangyao, Mr. Lin Beixi and Mr. Zhang Guihang. In their hands: Specimens of *T. gigas* hunted down by Mr. Liang.

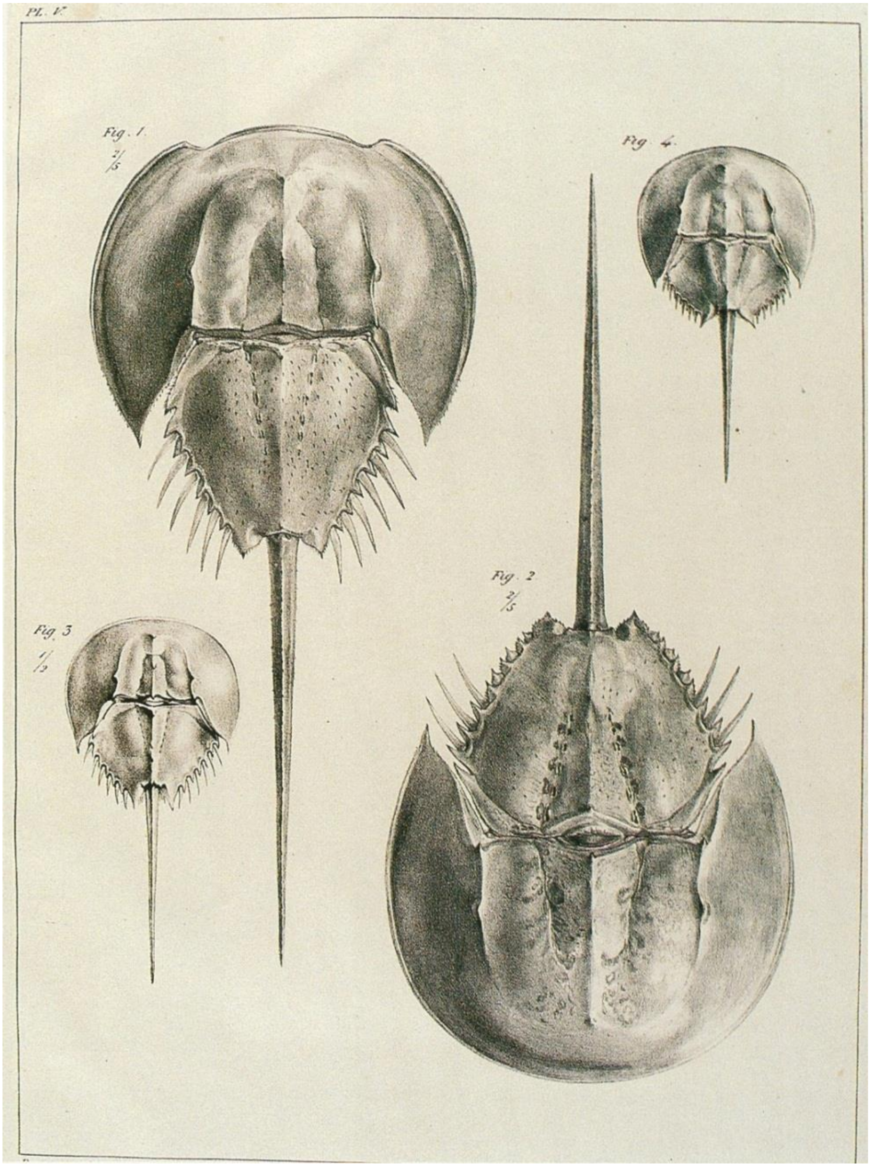


An adult female of *T. tridentatus* (average size for Beihai waters) surrounded by five apparently mature females of *T. gigas?* Or possibly a new (sub) species?

A close-up ventral and dorsal view of one of the five females pictured above. *Tachypleus gigas* or...?

Based upon the material available I cannot identify these eight females as either *T. tridentatus* or *T. gigas,* but on the other hand it would be premature at the present time to suggest that they represent a new species of *Tachypleus.* I have compared the eight specimens to Figures 3 and 4 in the manuscript drawing from van der Hoeven's book published in 1838 and found a certain degree of similarity. van der Hoeven describes Figure 3 as "A small *Limulus (Tachypleus)* from Japanese seas", and Figure 4 is "A small *Limulus (Tachypleus)* from Chinese waters". In Figures 1 and 2 van der Hoeven depicts the large male and female of *Limulus longispina* which according to the classification of Pocock is identical with *Tachypleus tridentatus.* It is obvious from van der Hoeven's text to the illustration that he considers *L. longispina* to be dissimilar to the two small specimens that "may be *Limulus kabuto-gani".* But it is also a fact that today we have only one species of horseshoe crab in Japanese waters, namely *T. tridentatus* which many authors, including this one, refer to as *L. kabuto-gani.*

Are the two specimens in Figures 3 and 4 from van der Hoeven juveniles of *T. gigas? The* long, slender lateral movable spines in figure 3 could be a feature of *T. gigas,* whereas the shorter, stouter lateral movable spines on the opisthosoma of the specimen depicted in figure 4 are more like the conditions found in *C. rotundicauda,* but the telson is quite clearly triangular in section, ruling out *C. rotundicauda* immediately. Figures 3 and 4 are both drawn to half their natural size, which does indeed make them small. I find this a quite intriguing puzzle and would like very much to have the opinion of Japanese and other scientists about these questions.



Another beautiful illustration from J. van der Hoeven, 1838

Fig. 1: *Limulus longispina (T. tridentatus)* male.

Fig. 2: Female of the same species.

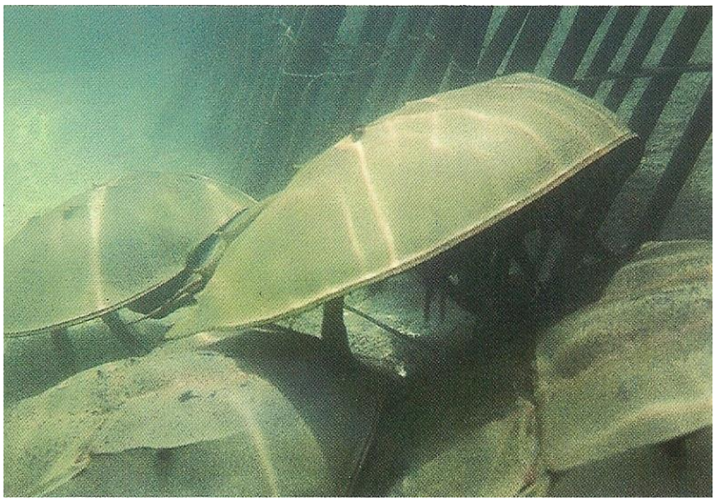
Fig. 3: A small *Limulus (Tachypleus)* from Japanese seas.

Fig. 4: A small *Stimulus (Tachypleus)* from Chinese waters.

According to Hoeven, the two latter species may be *Limulus Kabuto-gani (crabe à casque),* also known as *Umi-do-game* in Japan and *Unkiu* in China.

A cluster of males of *T. tridentatus* surrounding a female of the same species. (Beihai, China)

Two males of *T. tridentatus* climbing an underwater fence. (Beihai, China)



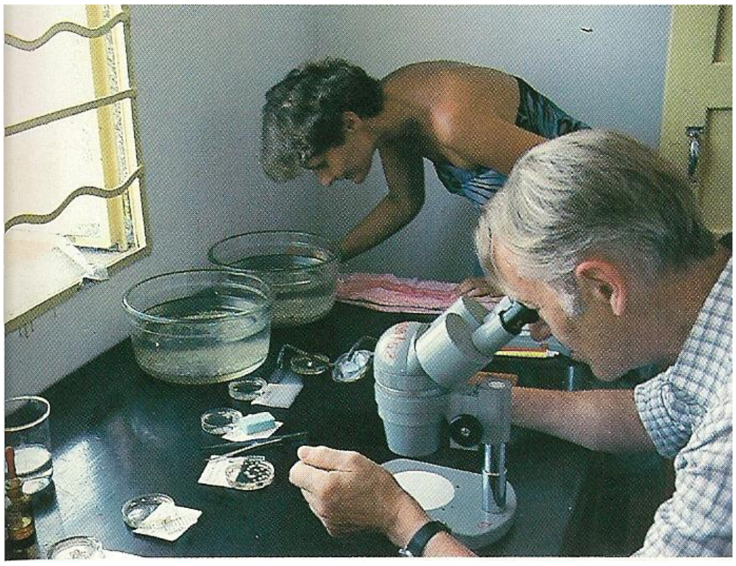
T. *tridentatus* during the rush-hour in Beihai, China.

Mass mortality following an unusually warm spell in Beihai. The dead *tridentatus* will not be wasted, but crushed and used as fertilizer.

Finally, it should be noted, that local fishermen in the vicinity of Beihai City know about *four* species in Beibu Gulf: Tiger horseshoe crab *(T. gigas),* Devil or Ghost horseshoe crab *(C. rotundicauda),* Eastern horseshoe crab fish or three spine horseshoe crab *(T. tridentatus)* and Yellow horseshoe crab (?).

Spawning begins at new moon and full moon and lasts for about a month from the middle of July on, but may vary somewhat from year to year according to climatic conditions.

T. tridentatus prefers to lay its eggs in rather coarse sand between the upper and lower tide lines, and if it cannot find an open sandy beach it may spawn at the bases of rocks or embankments where sand occurs only in limited areas. In Japan, apparently, no egg laying will take place even on a suitable sand beach if there is no mud flat in the vicinity, and conversely no small adolescents are found on mud flats if there is no sand beach nearby suitable for egg laying.



The author's wife, nursing teacher Kirsten Mikkelsen, and Professor, Dr. Med. Viggo Faber, Rigshospitalet, Copenhagen, examine eggs and larvae of *T. tridentatus* in the laboratories of Professor Tsai Singyi, Dept. of Oceanography, Xiamen University.

During oviposition the spawning couples may be covered by sea water. The couple proceeds forwards as the eggs are being laid by the female in a number of batches, each marked by a miniature sand mound. Each of these mounds measures 30-40 cm in diameter. One series of these mounds frequently extends 4 metres (most often 2 metres) and may display up to 12 mounds, 10-15 cm apart. Each mound marks the spot where an egg mass 4-7 cm in diameter can be found approximately 15 cm below the surface. The number of eggs per female is about 20000 and each nest may contain up to 1000 eggs.

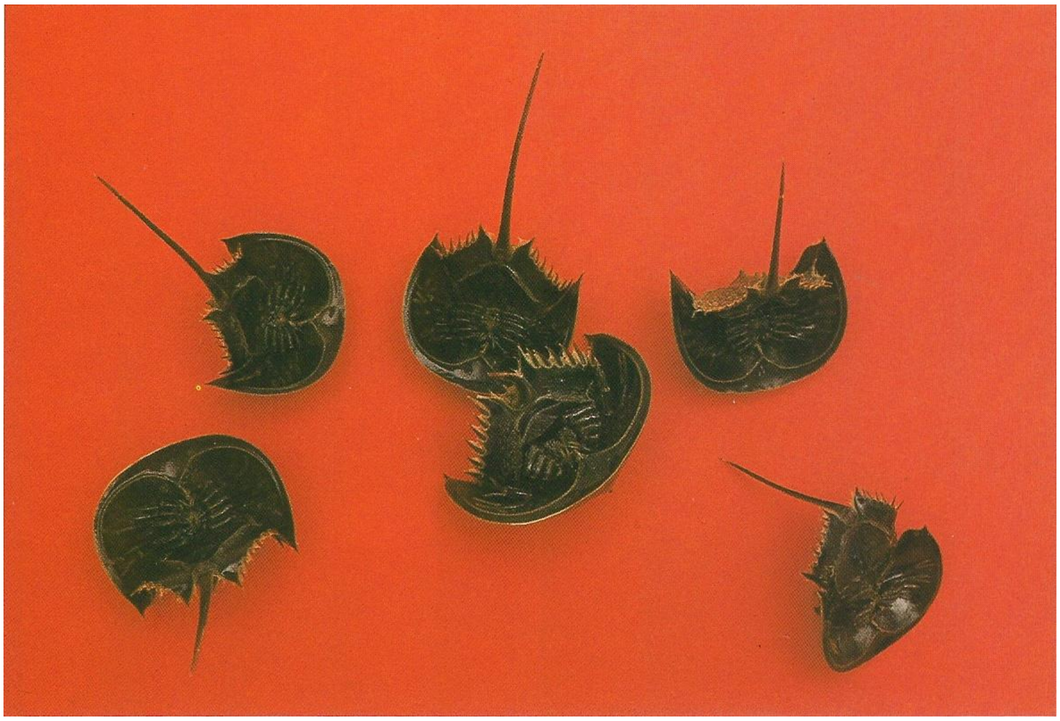
Series of mounds containing no eggs have also been encountered—although apparently the mounds have not been disturbed.

The newly laid eggs are white with a greenish tinge, more or less polyhedral in shape (owing to mutual pressure) and about 3 to 3.3 mm in diameter.

Approximately five weeks after fertilization the eggs have grown to full size (i.e. a diameter of 5.1 to 5.5 mm), and hatching takes place by the end of the fifth week and during the sixth. Towards the end of the sixth week all the trilobite larvae will be out in the sand, where they remain piled together in the nest. In this trilobite stage the animals have a total body length of 7.4 mm and the colour of the cuticle is white tinted with a shade of yellow.

It appears that a young *T. tridentatus* spends its first winter in the sand in the trilobite stage and moves to the water very early next spring.

Eggs, larvae and newly emerged hatchlings of *T. tridentatus* (formalin-fixation) donated to the author by Professor Tsai Singyi, Dept. of Oceanography, Xiamen University, Fujian Province, China.



Juveniles of *T. tridentatus* from Beihai. Natural size 7 to 15 cm.

Juveniles of *T. tridentatus* in the careful hands of Mr. Liang Guangyao on the terrace outside Guangxi Institute of Oceanography, Beihai, China.

The characteristic frontal margin, or flange, of the male *T. tridentatus.*

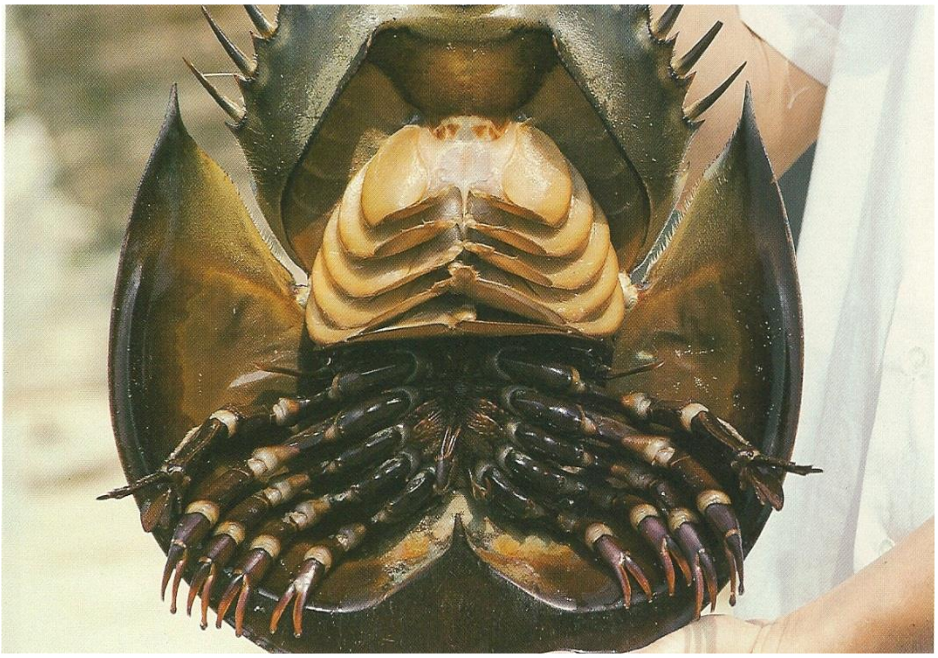
(Dongshan Island, Fujian province, China)

Adult female of *T. tridentatus*
(Xiamen, China)

The appendages of a fully mature female *T. tridentatus.*

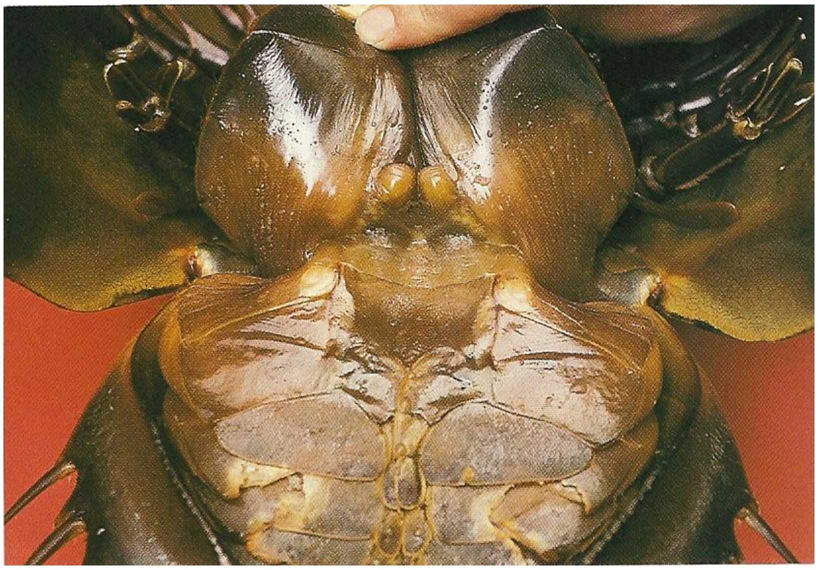
Normally shielded by the carapace but here fully exposed: The appendages of a male *T. tridentatus.* Note also the frontal "arch" of the flange, characteristic for males of this species.

A male *T. tridentatus* presenting itself for anatomical studies. (Xiamen, China)



An adult female of *T. tridentatus* hanging upside - down in an effort to display details of the ventral anatomy. (Xiamen)

A "portable" *T. tridentatus* female, later served for our supper.



Gill books and genital area of a female *T. tridentatus.* (Xiamen)

Gill books and genital area of a male *T. tridentatus.* (Xiamen)

At the appropriate spring high tide the young migrate to mud flats adjacent to their natal beaches, where they pass the early period of their life. The smallest individuals found on the mud flats had body lengths of 8.8 mm and were already of a darker color than in the trilobite stage. The next smallest specimens had body lengths of 11,3 mm and could not be distinguished from adolescents on the basis of their external morphology.

Ecdysis in *T. tridentatus* occurs during the summer only and most frequently in late July, when exuviae are found in large numbers and adolescents in the process may frequently be observed.

When the body length has reached 220 mm the sexes can often be distinguished by the presence of species specific indentations in the anterior portion of the rim of the male and the much shortened, fourth-to-sixth, movable opisthosomal spines of the female. Apparently the females attain their final form one year later than the males.

A female *T. tridentatus* painted by the artist Mr. Wu from Nanning, China.

Apart from the sex differences mentioned above, mature males may of course be easily distinguished from mature females by their monodactylid second and third prosomal appendages used for clasping the opisthosoma of the female. In immature individuals, however, these differential characteristics are absent and one is compelled to rely on other characteristics to distinguish the sexes. In fact, it is quite easy to mistake a male for a female and vice versa. Help is at hand by turning the animal ventral side up and study the form of the genital opening and its adjoining part. In the male the genital aperture is circular and lies on a relatively large papilla while in the female it is simply slit-like.

Individuals longer than 15 cm in body length (excluding the telson) live off the shore and are only caught in fishermen's net during the warm periods of the year, especially when they migrate from deep areas of the sea towards the breeding beaches.

Sunset over Clearwater Beach, Hong Kong.

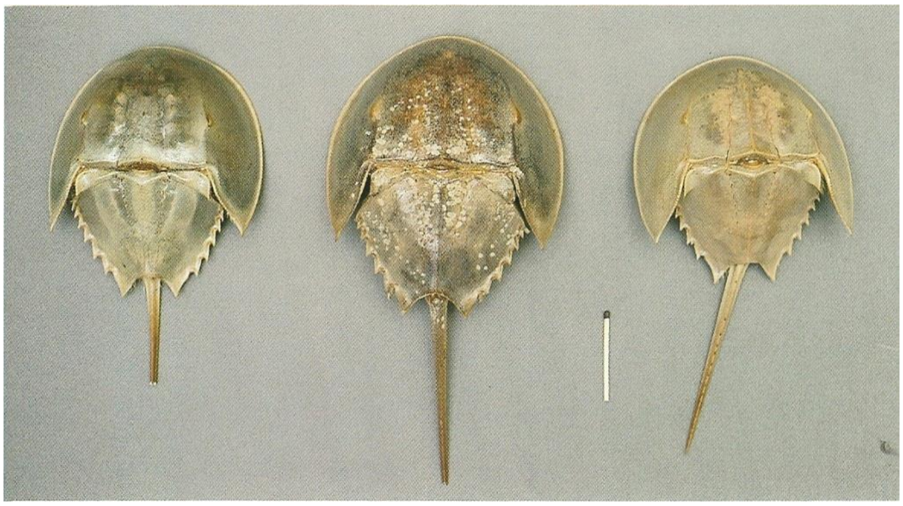
One of the largest specimens reported from Japan was a female 70 cm long including the telson.

The largest female I have ever seen was a Chinese specimen weighing 7.9 kilograms when caught and measuring 81 cm including the telson. From time to time during the past ten years information has reached me from various sources about horseshoe crabs occurring along coastlines where they were not supposed to be.

In the Mediterranean Sea such localities include the east coast of Rhodes, the Aegean Sea northwest of Athens, the islands of Corfu and Cyprus, and off the small town of Bozburun in Turkey.

The African Mystery

From the coastlines of Mauritania, Senegal and Gambia in West Africa reports about the presence of horseshoe crabs have reached me through a Danish friend living in the Canary Islands. Very recently it appears that a Spanish marine biologist has identified the living fossils on the east coast of Lanzarote.



From left to right: A male *Tachypleus gigas* from the Moluccas. A male *Limulus polyphenus* caught off the west African coast, and a female *L. polyphemus* from the Florida Keys.

Dorsal view of horseshoe crabs bought from dealers in Elat, Israel. Closely resembling specimens - if not actual examples - of the American *L. polyphemus.*

Altogether 24 horseshoe crab specimens were brought home from Elat, Israel. All males and all comparatively small. Local expertise of the highest international reputation deny their natural occurrence in Israeli waters.

And, finally, interesting and concurrent information about the occurrence of horseshoe crabs has reached me from Elat in the southernmost part of Israel, from the Aqaba Gulf along the east coast of the Sinai Desert and from the Red Sea coastlines of Sudan and Ethiopia.

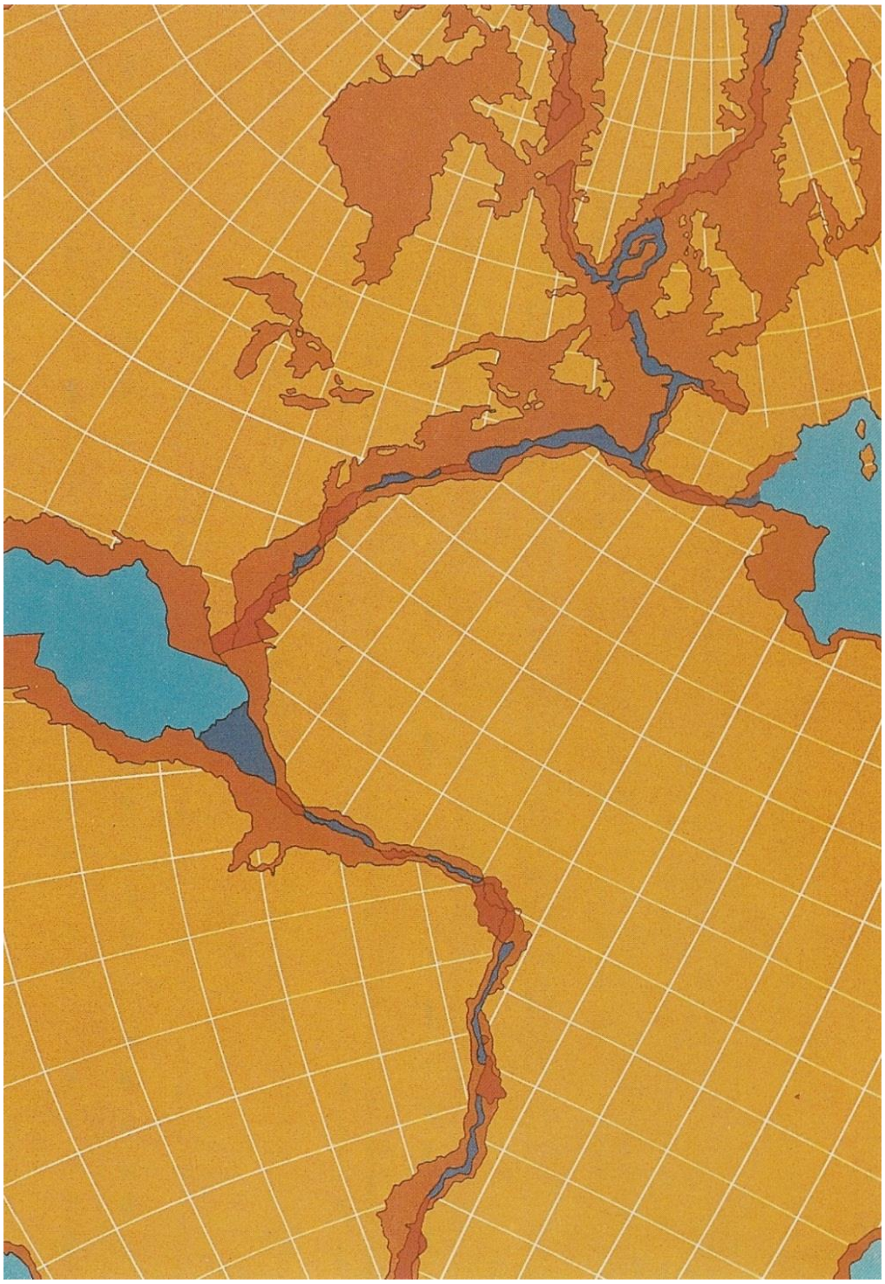
Before one starts to hunt horseshoe crab "ghosts" all over the world it is an excellent idea to study an article written in 1977 by the Danish zoologist Dr. Torben Wolff: "The Horseshoe Crab *(Limulus polyphemus)* in North European Waters".

The article lists 18 cases where *Limulus polyphemus* has been found in North European, mainly Danish, waters between 1968 and 1976. Three of the specimens were alive, and most of the remaining crabs were in a fresh state when they were caught.

From tagging experiments we know that an adult *Limulus polyphemus* will migrate a maximum of 35-40 kilometres during its lifetime, and with just a superficial knowledge about its capabilities as a swimmer we can rule out the suggestion that *Limulus* should be able to cross the Atlantic Ocean on its own.

We also know that adolescents and adults of all species are remarkably hardy and that they can be shipped long distances alive. The adults can survive for many days out of water, even without food, provided that their gill books are kept moist.

For these and other reasons it was concluded that all specimens found in North European waters had been collected on the east coast of North America and carried across the Atlantic alive on East European trawlers from which they had been thrown overboard *en route* to the home ports. It is claimed that East European fishermen are notoriously fond of releasing a variety of marine animals in habitats where they do not naturally occur.



Presumed position of the continents until about 65 million years ago.

Note the comparatively narrow water separating the east coast of the United States from the west coast of Africa. And note also the open connection to parts of Europe where fossilized horseshoe crabs have been found in abundance.

I, too, love America, but why should all the horseshoe crabs have chosen American citizenship when the North American continent drifted westwards?

Other apparently stray specimens of *Limulus polyphemus* have been found off the Terschelling Island, on the coast of North Wales, off the Farallon Island west of San Francisco and, finally, a small specimen was found on the Great Barrier Island, 100 km north east of Auckland, New Zealand. A live specimen of *Carcinoscorpius rotundicauda* has been caught off the Isle of Wight, and another C *rotundicauda* was found clinging to the stone fencing on a dock in Auckland harbour in 1908.

Having recognized the logical explanation for most of the incidents of stray horseshoe crabs listed above, we still find it tempting, however, to think that niches of naturally occurring horseshoe crab populations might have been overlooked somewhere in the world far from their officially designated habitats.

Considering that the east coast of North America is believed to have been positioned close to the West African coastline - with Miami not far from Dakar, and Maine only a short distance from Morocco - until about 65 million years ago, and assuming that the living fossils inhabited the comparatively narrow water separating the two huge continents, is it not plausible that some of the horseshoe crabs decided to remain on the African coast when the two continents started drifting apart?

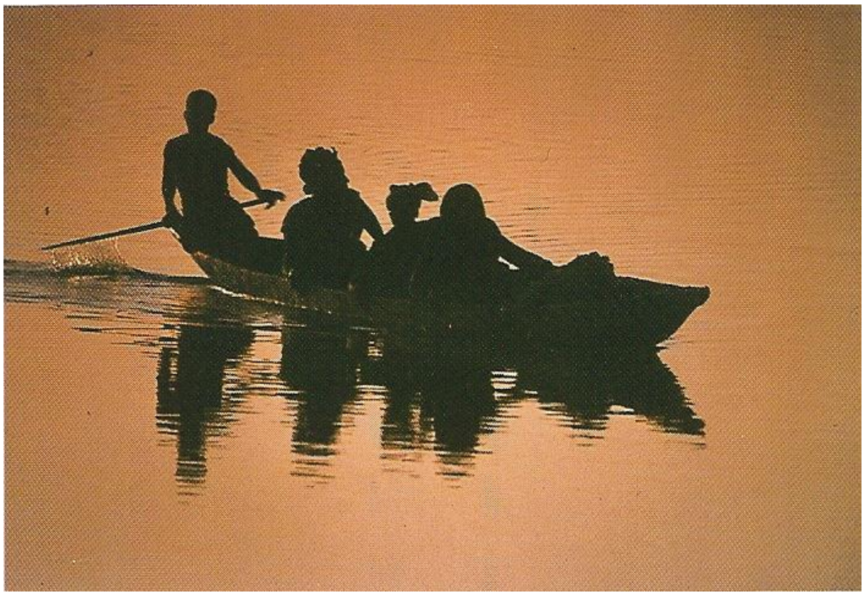
And when the Canary Islands were ripped out of the African continent around Sidi Ifni on the Moroccan coast, is it not possible that a population of horseshoe crabs followed these islands?

Based upon numerous reliable reports from scuba divers, fishermen, tourists and friends living and working in these areas, and having obtained a number of well-preserved horseshoe crabs allegedly caught *on location,* we finally undertook a series of expeditions to two destinations of major interest, namely the Canary Islands-West Africa and the Elat/Aqaba Gulf and the Red Sea in search of such discreet horseshoe crab populations.

Until about 1976 Korean trawlers fishing along the coast of West Africa landed and sold their catch in Las Palmas on the Canary Islands. Apparently, horseshoe crabs were a frequent by-catch because the Korean fishermen sold hundreds of dried specimens to tourists and entrepreneurs in the tourist industry. Around 1976 this fishing by Korean trawlers was stopped and the supply of living fossils soon dried out, too.

I was presented with one of these horseshoe crab specimens and could immediately identify it as the American *Limulus polyphemus.* As the Korean trawlers in question never crossed the Atlantic Ocean and never went anywhere near the east coast of North America, I would have been less surprised if I had been presented with a piece of Korean "homework": A specimen of one of the Oriental species picked up on the way to West Africa.

Local Spanish fishermen on the Canary Islands supported the Korean statements and told us about horseshoe crabs caught off the coasts of Mauritania and Gambia when they had fished for lobsters by means of huge traps.



River Gambia in the sunset.

We went to Dakar in Senegal with our diving equipment, and armed with piles of pictures of horseshoe crabs we searched the coastline and fishing villages from Cap Vert to the Sine-Saloum Delta. We crossed the border into Gambia and travelled up the fascinating Gambia River as far as Tendaba and Mansa Konko 150 kilometres from the Atlantic Ocean where the river is still brackish, bearing in mind that *Carcinoscorpius rotundicauda* lives 90 miles up the Hoogly River. We searched the Gambian fishing villages of Brufut, Gunjun and Tanji and interviewed hundreds of fishermen and other people on our way. During all the interviews we were very careful not to ask leading questions and when presenting the pictures we only volunteered the information that it was a crab-like animal with a hard carapace and a rigid tailspine - not to be mistaken for a ray.

The African moon is rising, near Tanji, Gambia.

In the fishing village of Fadiouth, a young fisherman immediately recognized the horseshoe crab and told us that he had had 5-6 specimens in his net the previous week at a depth of approximately 40 metres. The animals had had a total length of about 45 cm and he had dutifully cursed them before throwing them back into the sea because "they always get entangled in the fishing net and tear it up". If we really wanted to find horseshoe crabs, he advised us to continue one day's journey further south, but he added that during this cold season (January) the horseshoe crabs could only be found in relatively deep water.

In the fishing village of N'Gor, on the tip of Cap Vert, we met a shipbuilder who had trained in Portsmouth and spoke excellent English and French. He explained that for generations the fishermen had used the scooped out carapace of the horseshoe crabs for bailing in the pirogues, but now plastic containers had taken their place. According to this shipbuilder, the rigid telson is still used by some to wind up the fishing line, and in the bow of the old pirogues one may still find a small quiver containing amulets like shark teeth and the characteristic anterior claws of a male horseshoe crab, which he quite aptly compared to a thumb. He even knew about the morphological differences between the second pair of prosomal appendages in males and females.

Another convincing statement came from a young Gambian fisherman whom we met at the fish market in Bakau. He enjoyed the esteem of his elders because he had been signed on as an assistant on a big trawler fishing off the coast of Canada and the United States. He had seen a lot of horseshoe crabs "over there", and he had also seen a lot of horseshoe crabs when fishing along the Mauritanian coastline, and, especially, around Agadir in Morocco. Invariably, the horseshoe crabs were immediately thrown back into the sea—they are considered useless creatures.

In Bel Air, close to Dakar's industrial harbor, we finally met Mr. Youssu Ka. I made a loose sketch in the wet sand and without the slightest hesitation he told us about this 'calabash with tail and many legs", indeed a short and accurate description of a living fossil.

After this description we showed him pictures of the ventral and dorsal side of *Limulus polyphemus,* and there was still absolutely no doubt in his mind. He explained to us that this animal has no specific name among the fishermen and that it only lives in special areas where the water is shallow and the bottom muddy and grassy. Youssu Ka even had the impression that they lived on mud because they buried themselves completely into the substratum during the day, and were only active late in the afternoon and at night.

I had to return from Africa without any living or dead evidence to support my theories about horseshoe crab niches in West Africa or in the Canary Islands. But one day I will return to these beautiful beaches and picturesque fishing-hamlets and continue the search.

On the same basis of circumstantial evidence as obtained from West Africa and the Canary Islands, supplemented by two dozen small, well-preserved males of *Limulus polyphemus* mostly bought from various shell dealers in Elat, we finally decided, in 1985, to take a closer look at the Aqaba Gulf and certain coastal areas of the Red Sea.

The beautiful coral world a few hundred metres off Nahalat-el-Tel in the Sinai Desert.

I was aware that back in 1977 Israeli scientists had carried out rearing experiments with *Limulus polyphemus* in the mariculture laboratory attached to the famous underwater observatory 7 kin south of the city of Elate. And it was of course possible that at least some of the 24 specimens obtained in Elat had come one way or another from these mariculture ponds.

I kept thinking about the work by Dr. Wolff mentioned above in which he refers to the article written by Lloyd in 1874 giving the following explanation for the finding of 5 *limuli* on the Dutch coast in the summer of 1873: "In the 1860's large numbers of *Limulus* were imported by Lloyd and others from New York to Hamburg. Since not all could be sold to aquaria he persuaded a sailor to release "a cabful of crabs" in saltwater beyond Helgoland, on the way from Hamburg to London. This took place in August 1866, and some crabs must thus have survived for at least seven years in the area".

Were the Elat-specimens survivors from the mariculture experiments? The shell-dealers insisted that the specimens were provided from time to time by Bedouins, and the Bedouins insisted that they had found the animals dead on the beaches of the Sinai Peninsula not only south of Taba, but as far south as possible: Ras Muhammad, and also on the western side of the peninsula bordering the Gulf of Suez (Et Tur).

From personal friends and connections, several of them scuba divers, we had reliable reports of live horseshoe crabs having been observed around the famous shipwreck "Maria Schröder" north of Shora el Manqata. Three mating pairs of horseshoe crabs had been seen just off Nabq and other sightings included Nahalat-el-Tel and Ras Nasrani along the Gulf of Aqaba.

Furthermore, we had several independent reports about horseshoe crabs existing near Hurghada in Egypt, south of Port Sudan (Trinkitat) in Sudan, around Mesewa in the Mesewa Channel in Ethiopia and in the southernmost part of the Red Sea, Aseb Bay behind (and sheltered by) Haleb Island virtually on the border between Ethiopia and Djibouti.

But all our efforts were in vain - we didn't find one single horseshoe crab, dead or alive, except in Elat.

Many statements about their existence have reached me since our return. I have left preserved specimens all along our trail, so maybe one day we shall have to go back to compare the preserved specimens with live ones caught by local fishermen ...

PART TWO

THE SECRET IN THE BLUE BLOOD

Killer Microbes and Defense Mechanisms

Evolution, in the biochemical sense, consists largely in the progressive substitution of one amino acid or nucleoside for another. But the manner of assembly of the original monomeric building blocs: amino acids, sugars, phosphates, organic bases and lipids into the biologically active polymers: proteins (enzymes), nucleic acids and cell membranes still remains somewhat obscure, just as it is unknown why the present set of 20 amino acids, which are sufficient to build proteins and process genetic information, was selected.

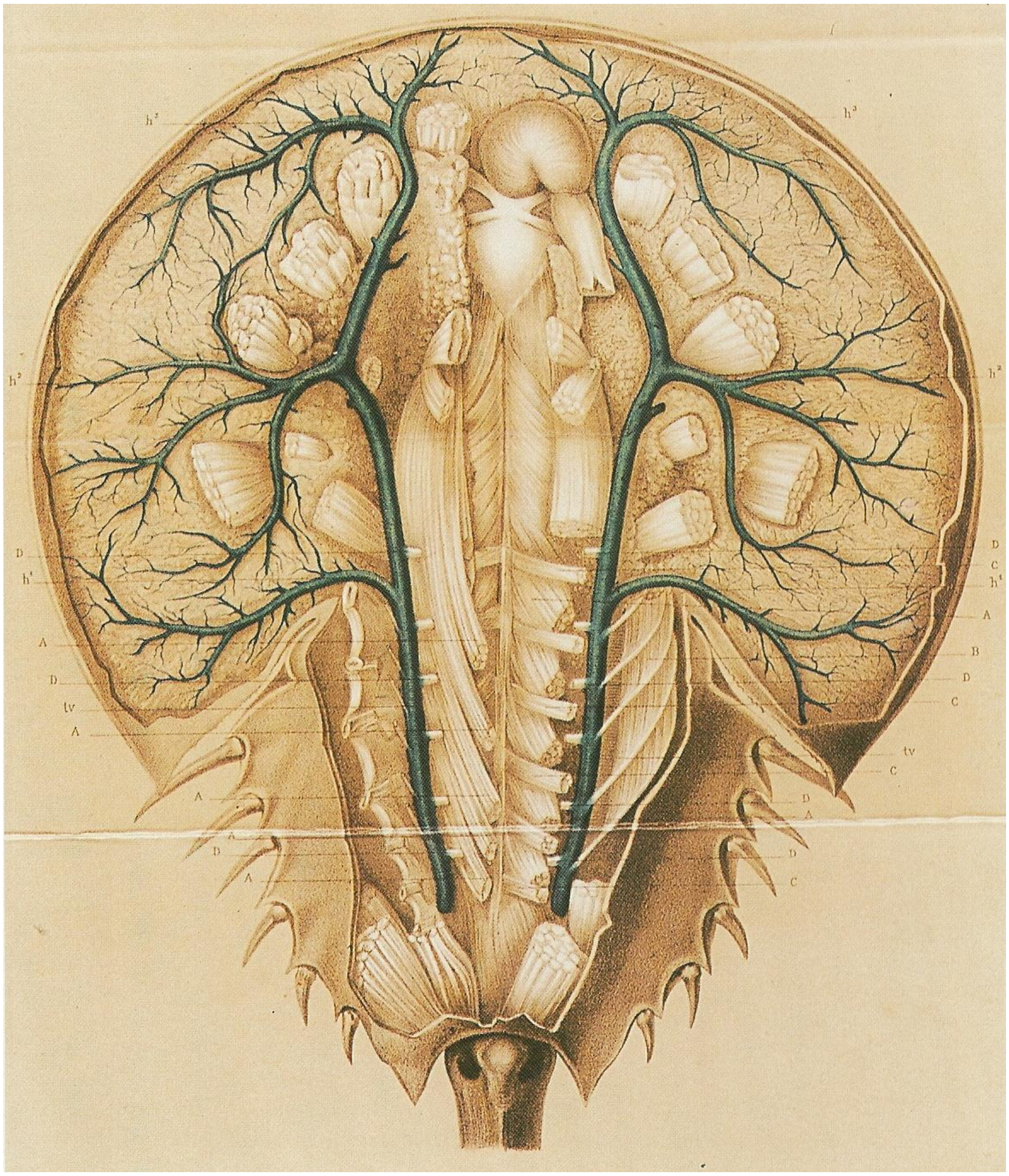
No living organism is identical biochemically with its antecedents, but components of earlier biochemistries have been preserved, reasonably including some of the antigenic properties characteristic of present day viruses, bacteria and blue-green algae (cyanobacteria).

If we return to the Palaeozoic *terra firma,* that is, *Pangaea* , and imagine ourselves standing breathless in the reducing atmosphere, we are in any case looking in vain for macroscopic, terrestrial life. Had Leeuwenhoek been there he might have been able to find soil bacteria, fungi and, I suppose, lichens, too, but all sizable metazoan life is marine, although the sea may still be fresh.

The emerging eukaryotes were all forced to adapt to the biosphere and physical environment prevailing in and along the coastlines of the Tethys Sea. The energy to support the early marine animals was supplied by photosynthesizing algae. Besides contributing detritus to the invertebrate community, these early members of kingdom metaphyta may quite likely have been grazed directly.

These external conditions also provided an essential impetus to the development of internal defense mechanisms against the prokaryotes which by then had been the sole inhabitants on the planet for approximately 2.5 billion years. (According to fascinating hypotheses, the eukaryotic progenitor could have been the result of a remarkable degree of symbiosis, namely a conglomerate - or fusion - of prokaryotes into some kind of amoeboid organism, suggesting that cellular organelles like mitochondria and chloroplasts were originally free-living microorganisms).

In all living organisms, the defense of bodily integrity encompasses a certain degree of ecto - and endosymbiosis. As phylogenetic groups of organisms appear they tend to spread and occupy all available ecological niches of the physical environment. Their bodies in turn constitute new ecological biohabitats ready for occupation by those species that possess the potential and capability of adapting to them.



The Secret in the Blue Blood.

Three degrees of symbiotic relationships are generally recognized: *mutualism, commensalism* and *parasitism.* Lichens have long been taken as a standard example of true symbiosis where the fungus benefits since it is wholly dependent on the alga for food, just as some lichen fungi produce substances necessary to the growth of the "enslaved" algae.

All three degrees of symbiotic relationship, together with principles of antibiosis and antagonism, were probably well developed in the neritic communities, and were thus "merely" further developed and exploited by the terrestrial communities in the eternal efforts to maintain ecolibrium in the biosphere of the planet Earth.

Our present knowledge about the sometimes abundant epiphytic viruses and bacteria adhering to the perialgal slimelayers (probably vital to the seasonal decomposition of algal blooms) and our knowledge about the very potent toxins produced especially by the blue green algae themselves suggests that some humoral and cellular defense mechanisms must have evolved in order to meet the potentially pathogenic or even lethal sequilae of grazing and other feeding habits, and, of course, also to defend the invertebrate organisms against the possible consequences of bodily injury, microbial invasion and the establishment of infection.

Before presenting some of the invertebrate principles of defense, we must note that antibacterial activity has been demonstrated in extracts from at least 42 species of lichens, of which 27 were active against *Staphylococcus aureus* and *Bacillus subtilis.* At least four species of lichens inhibit growth of *Pseudomonas vulgaris* and *Alcaligenes fecalis,* while none of the 42 species examined showed antagonism against *Escherichia coli.* But fungal antibiotics against *E. coli* and most other Gram-negative bacteria were isolated from *Streptomyces griseus,* just as *Penicillium notatum* and *P. chrysogenum* extracts have been demonstrated to be effective against most Gram-positive bacteria.

A substance named *cephalosporin,* with similar antibiotic properties as the penicillins, has been isolated from a mold of the genus *Cephalosporium.*

Antibiotics produced by the genus *Streptomyces,* a sporulating group of *Actinomycetes,* comprise e.g. novobiocin from *S. niveus* and erythromycin from *S. erythreus.*
The most potent antibiotics that we know to be active against fungi are the griseofulvins synthesised by several species of *Penicillium,* and polyenes produced by various *Streptomyces* species.

And, of course, the lowest of the invertebrates, the sponges, also produce substances possessing antimicrobial properties, ranging from general inhibition of a variety of naturally occurring sea water bacteria to the rather specific *ectyonin* produced by the North Atlantic "red beard" sponge, *Microciona prolifera,* which inhibits growth of the "imperfect" fungus *Candida albicans.* In a few minutes we shall return to the sponges since they have other capabilities of significant immunological importance, but right now we will jump from Palaeozoic puzzles and approximately 550 million years ahead in time, in order to attend the 13th International Physiological Congress in Boston in 1929, where the famous Danish physiologist August Krogh is about to make a speech. One important sentence will be this: "For a large number of problems, there will be some animal of choice, or a few such animals, on which they can be most conveniently studied".

This brilliant idea had actually occurred to Metchnikoff some forty years earlier, when he inserted rose thorns into the body cavity of sea star larvae and observed, some 24 hours later, how mobile hemocytes (blood amoebocytes) flocked around the thorns. Metchnikoff rushed to Messina in order to present his observations to the famous professor Virchow, who immediately recognized and appreciated the importance of the experiments and results. Names for the effector cells, phagocytes (Gk. *phagein,* to eat), and for the principle, phagocytosis, were provided by a friend, Professor Claus, in Vienna, who looked them up in a dictionary of zoology.

Back again, in lower Cambrium, the unicellular protozoa have already applied phagocytosis, not only to prevent the invasion and growth of potentially pathogenic microorganisms, but also as a means of ingestion and digestion of food.

Amoebae move by the formation and retraction of pseudopodia, and the fact that an amoeba never phagocytoses its own pseudopodia, or those of other amoebae, is the first – feeble - expression of the fundamental principle in immunobiology: The distinction between "self" and "non-self".

One step up the evolutionary ladder we visit again the first diploblastic metazoa belonging to phylum Porifera, whose members lay the foundation of cellular differentiation and specialization. For instance the marine sponge (they are all marine except one species) *Terpios zeteki* contains five types of free mesogloeal cells with phagocytosing abilities. Most significant among them are the *archaeocytes,* which by hypereutrophication may engulf particles larger than the cells themselves. The *archaeocytes* are assumed to be the first distinct phagocytes.

Experiments with the sponges *Haliciona* and *Microciona* have demonstrated that in both species, auto- and isografts, even from individuals from distant localities, were readily accepted, but grafts from foreign types were rapidly rejected.

Phylum Annelida, the internally and externally segmented worms with a closed circulatory system of elongated, branched vessels comprises approximately 8 700 extant species. Members of this phylum (e.g. the marine nereis, leeches - and earthworms appearing in Ordovicium among the earliest terrestrial invertebrates) as well as the other coelomate invertebrates contain granular and agranular, mononuclear hemocytes, one function of which is phagocytosis.

Within phylum Annelida, a distinct cell-mediated immunity has been demonstrated, evidenced by rejection of allotransplants, followed by a short immunological memory.

Rejection of allografts has also been reported from experiments with reef building corals, *Montipora verrucosa.*

Experiments with gorgonians *(Anthozoa)* have shown that if pieces of two different genera are brought together, there will be both failure of fusion and evidence of damaging interaction in the form of blistery tissue where contact occurs.

Moving up to phylum Mollusca, the ubiquitous soft-bodied animals comprising approximately two hundred thousand living species including chitons, snails, slugs, whelks, tooth shells, clams, mussels, oysters, squids and related cuttlefish and octopus, we encounter the invertebrate phylum which has attracted maybe the greatest immunological attention of all invertebrate groups (except *Limulidae* during the past decade, or so).

In this context I will concentrate my attention on the marine species, since the earliest evidence for terrestrial molluscs (the pulmonate snails and slugs) appears as late as in the Carboniferous period.

Phagocytosis, encapsulation, and necrotization are the primary functions of molluscan hemocytes. Foreign particles may either be engulfed by phagocytes and carried across epithelial layers away from vulnerable organs or tissues, or be degraded intracellularly by hydrolytic enzymes. In the Australian clam, *Tridacna maxima,* circulating phagocytosing macrophages will engulf injected particles in the course of few hours. Phagocytosis is usually rapid, and if ineffective, encapsulation occurs, effectively surrounding and isolating "non-self" material, which is subsequently slowly eliminated from the body. In the California sea hare, *Aplysia californica,* India ink injected intramuscularly into one foot will be completely cleared after four days. With carmine particles the reaction is different, since encapsulation results in the formation of large nodules that are walled off by a cellular capsule, visible even 30 days later.

Autochthonous (Gk. *autos, self - chthon,* ground) gills implanted in the adductor muscle of *Pecten maximus* are eventually isolated by encapsulation. However, auto-and allotransplants behave similarly and encapsulation is probably part of the organism's efforts to seal off the lesion rather than a recognition phenomenon.

Allografting of small portions of the mantle to slits in the connective tissue near the palps in *Crassostrea gigas* resulted in some allografts being rejected while others remained viable and appeared normal. In the gastropod mollusc *Australorbis glabratus* allogeneic grafts produced only a transient coelomocytic infiltration, while xenogeneic (Gk. *xenos,* strange-*genos*, descent) tissues from *Planorbarius corneus* provoked a marked cellular response, followed by graft destruction.

Although much remains to be known about the fate of soluble substances, it has been clearly demonstrated that oyster hemocytes can pinocytose (Gk. *piein,* to drink-*kytos*, hollow) mammalian and *Limulus polyphemus* serum protein both in vivo and in vitro.

Humoral immunity in marine molluscs is mediated by naturally occurring bactericidal and bacteriolytic substances, by naturally occurring and inducible agglutinins and by opsonic (Gk, *opsonein,* to cater) factors modulating and facilitating phagocytosis by hemocytes.

Naturally occurring agglutinins against several marine bacteria have been isolated and described in the sea hare, *Aplysia californica,* and experiments on the specificity of the agglutinin serum factors in *A. californica* showed that both vertebrate red blood cells and certain marine bacteria were agglutinated, but the terrestrial bacterium *Serratia marcescens* was not.

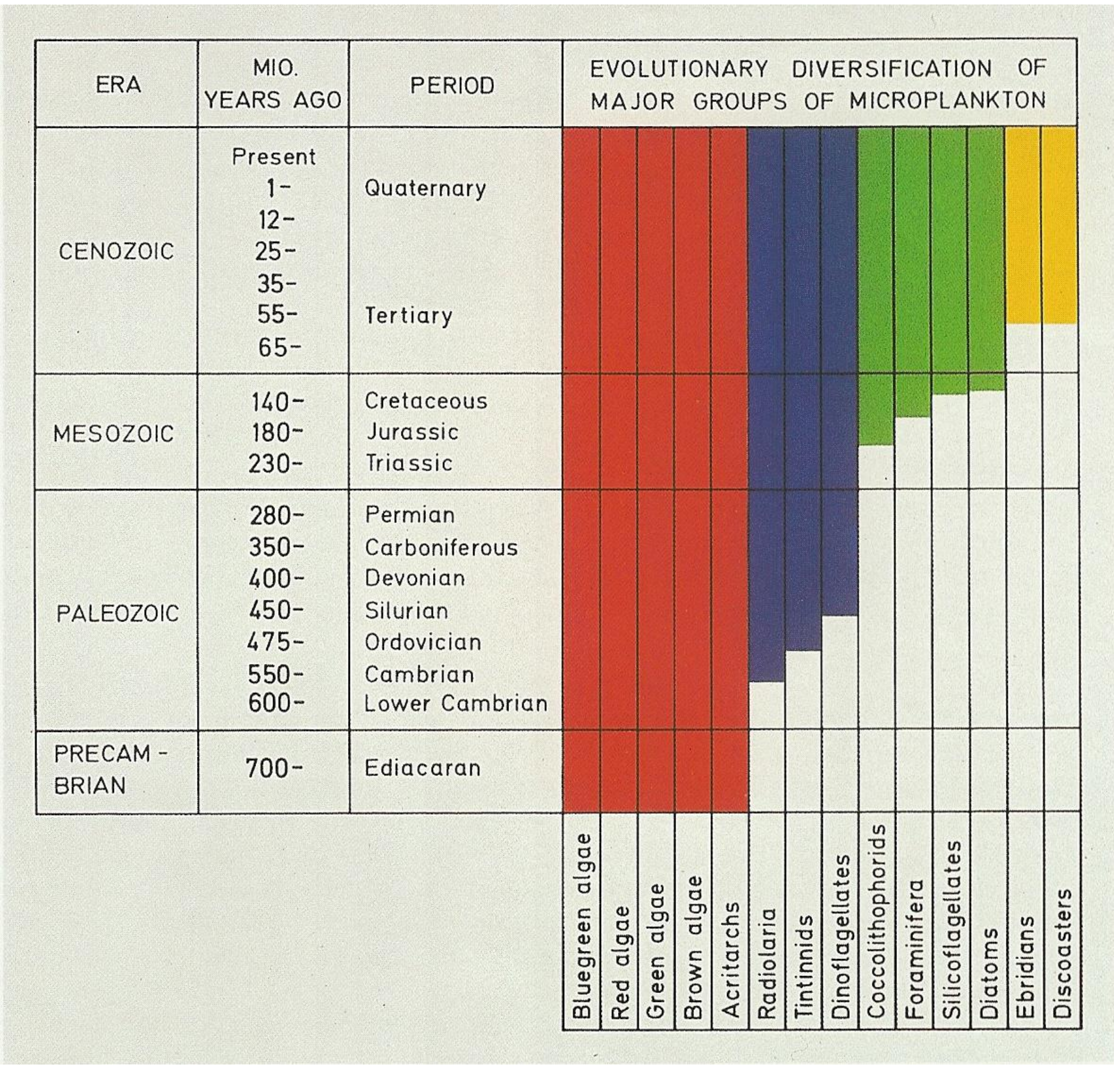
Several hemaggiutinins are present in the cardiac blood of *Crassostrea virginica.* Absorption studies with different erythrocytes have revealed various degrees of cross reactivity, suggesting the presence not only of several agglutinin types but also of different antigenic sites. The opsonic properties of these oyster hemolymph proteins manifested themselves both in an increase in the number of phagocytes that engulf one or more particles, and in an increase in the average number of particles per phagocyte.

Moreover, *C. virginica* hemolymph contains the bacteriolytic substance lysozyme, which has a lytic effect on suspensions of live *Bacillus inegaterium* and dead *Micrococcus lysodeikticus.* Lysozyme also has a well-known destructive effect on the mucopeptides contained in several Gram-positive bacteria.

This list of immunological principles and defense strategies to protect bodily integrity could be continued at length with fascinating examples of evolutionary adaptations - all designed and brought into efficient service long before the conquest of *terra firma* and, of course, hundreds of millions of years before the emergence of *Homo,* And with the emergence of *Homo scientificus* we have indeed learned much about infection and immunity from the study of the marine flora and fauna - as recommended by Professor August Krogh.

The scientific quest for understanding has resulted not only in important theoretical achievements but certainly also in the identification of a number of pharmacologically significant chemical constituents in marine flora and fauna.

Carrageenan is a compound derived from red algae, with the ability to lower serum lipids significantly when administered intravenously to human beings, and, in addition, it has been shown to prevent the development of atherosclerosis. With proper pre-treatment, carrageenan increases the calcium uptake in bone and reduces serum calcium, and furthermore carrageenan has been shown to inhibit the growth of certain types of influenza and mumps virus.



At the dawn of the lower Cambrian, the sea was - then, as now - teeming with microscopic algae. From the blue green algae, the red algae (Rhodophyceae) developed.

At first the lesser modified *Bangiophyceae,* which probably appeared well before the Cambrian era, either inland or in the relatively fresh Tethys Sea. Later, probably by or before the Ordovician period, the more advanced *Forideophyceae* developed and became the dominant offshore flora by Triassic time.

The red algae occur in all oceans today, but are most common in the tropical and warm-temperate regions of the southern hemisphere.

The coralline red algae have been active reef builders in the geological past, the fossils are abundant in strata of the Ordovician age. Typically, the marine *Rhodophyceae* are attached rather than free floating, and frequently they are epiphytic on other algae or parasitic on other red algae.

The green algae, found today in fresh and salt water, and in subaerial habitats with many epiphytic and a few epizoic species were also represented in the Tethys Sea, along with the brown algae *(Phaeophyceae)of* which nearly all known species are marine, although it is conceivable that the brown algae gave rise to a temporary terrestrial line.

The *Phaeophyceae* are especially common along or near the seashore in the cool parts of the world. They range in size from microscopic to a length of 150 feet, also comprising the tropical and subtropical genus Sargassum, known chiefly from the Sargasso Sea in the Atlantic Ocean.

By most people algae are considered a troublesome, annoying and potentially toxic pestilence - but they are also important additives in baked goods, beverages and pet food. They are used as culture medium (agar-agar) in applied microbiology, in the manufacture of rubber articles, linoleum and paints, in cosmetics and toothpaste, as fodder supplement for cattle and sheep, etc.

Alginates from brown algae have hemostatic effects, and alginate wool is used, for instance, in dentistry. Alginates may also be applied both for prevention and for the treatment of poisoning with radioactive strontium, since the extracts rich in guluronic acid residues have a high selective binding capacity for strontium.

A host of important compounds have been harvested from studies of the marine, invertebrate fauna.

The moult-inducing hormones, the ecdysones, were originally isolated from crustaceans. Sterols, reducing blood cholesterol levels have been isolated from sea-anemones, molluscs, bivalves and sponges. Saponins (holothurins) possessing antifungal activity, now used in anti-cancer chemotherapy, were found in sea cucumbers *(Holothuroideae),* while terpenes (crassin acetate) with bactericidal and bacteriostatic properties were isolated from gorgonians. Crassin acetate is also cytotoxic to certain cancer cells in culture. From gorgonians also prostaglandins have been isolated. These hormones are used for birth control, against peptic ulcers and asthma, and have a depressing effect on blood pressure besides their tranquilizing action on the central nervous system. From the sponge *Cryptotethys crypta* we got the structural model for synthesizing D-arabinofuranosylcytosine (Cytarabine), a compound that inhibits the growth of Sarcoma 180, Ehrlich carcinoma and L 1210 leukaemia in mice, and is effective in the treatment of Herpes simplex virus induced keratitis and acute myelogenous leukaemia in man.

Finally, we have discovered the compound Thelepin with antifungal properties and the well known insecticide, nereistoxin, in annelids.

Hundreds of similar stories about important discoveries emerging from experimental work with marine invertebrates can be told, but one story surpasses all others: *The story about the Limulus amoebocyte lysate test for the detection of endotoxin.*

The Secret in the Blue Blood

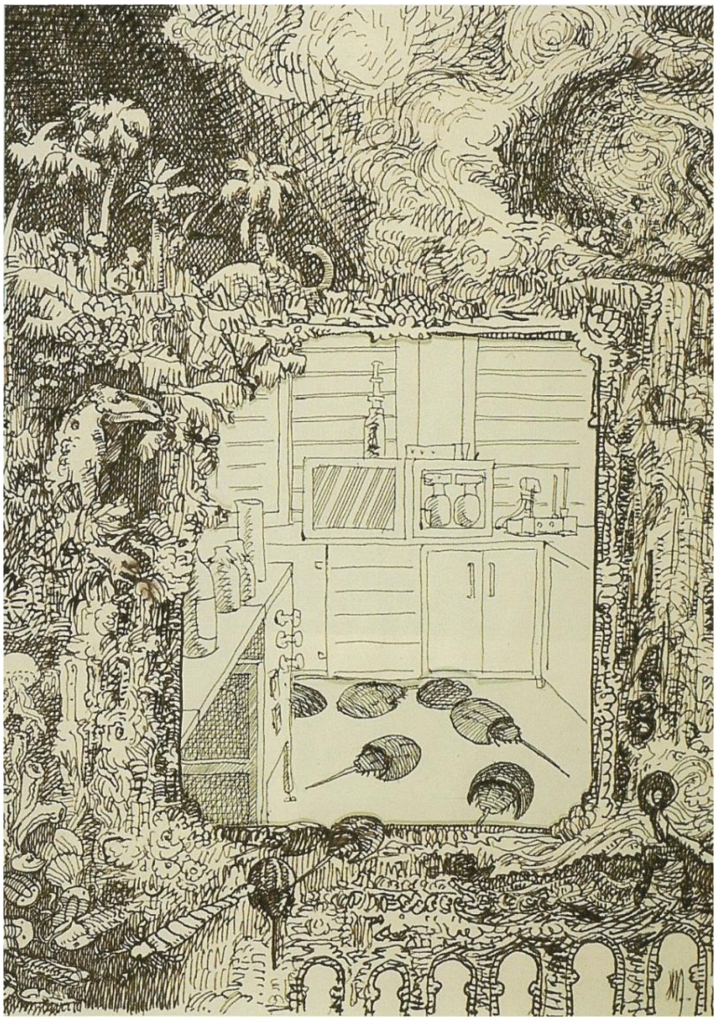
The American horseshoe crab acquired its scientific name *Limulus polyphemus* in 1758. The name refers to the large, oval, bulging eyes on either side of its prosoma *(litmus* is Latin for sidelong glance), and the two eyes, each the size of a pinhead and each equipped with a single lens, positioned closely together and low on the front of the prosoma – they are known as the median ocelli.

Although the ocelli are very small, they may be perceived to go back directly to *Polyphemus,* the enormous cyclops, son of *Poseidon,* who devoured some of *Ulysses's* companions, in return for which his median ocellus in the centre of his forehead was destroyed.

On close inspection, *Limulus* proves to possess nine eyes in all or rather, two pairs of eyes and five organs, sensitive to light, which are well hidden under the prosoma.

For more than 50 years, scientists have studied the lateral eyes of the horseshoe crab. Registrations of electric impulses from its optic nerve, made by Dr. H. Keffer Hartline among others, have revealed many fundamental physiological principles which can be transferred directly and generally to all visual systems.

Today, we know more about the lateral eyes of the horseshoe crab than about any other organ of perception of any other animal. The pioneer work earned Dr. Hartline a share of the Nobel Prize for 1976. Many scientific questions have been asked about this living fossil, and quite a few of the answers have been of considerable importance. One question, originally formulated by Dr. Frederik B. Bang in 1956, eventually produced an answer of extraordinary biomedical impact: The Limulus test for the detection of Gram-negative bacterial endotoxin.



In 1956 the living fossils began their triumphant march into modern biomedical laboratories.

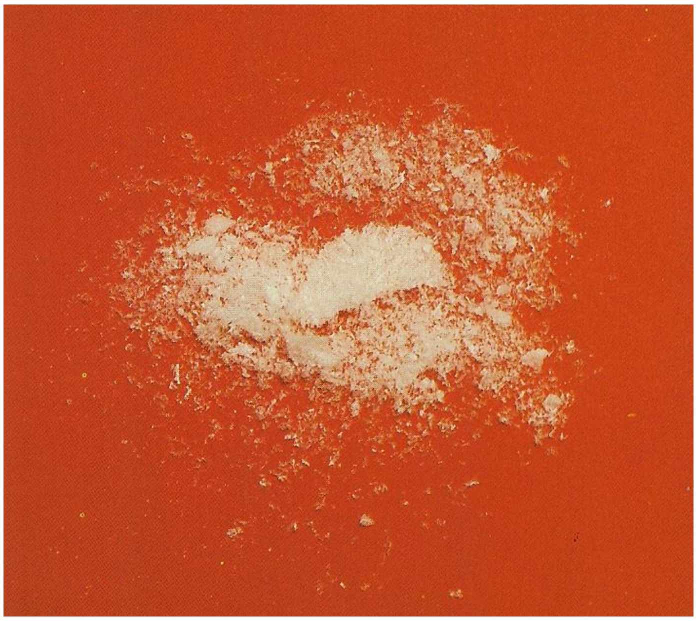
Endotoxins are found in Gram-negative bacteria as macromolecules composed of polysaccharide, lipids and smaller peptides (lipopoly-saccharide, LPS). They are part of the outer, triplelayered membrane of these bacteria, and not only do they assist in the selective transport of matter into the cell,they also participate in its defence against e.g. antibodies. When isolated from smooth bacterial strains the lipopolysaccharide characterizes the 0 antigenic specificity of the species, sero-group or sero-type of the bacteria in question, and it is the polysaccharide moiety that carries the antigenic determi-nants. Rough strains of Gram-negative bacteria also produce lipopolysaccharide, and simi-larly, the polysaccharide is responsible for the serologic R specificity.

The foetus in utero is sterile, but at birth the foetus is "contaminated" with the vaginal and fecal flora of the mother and other bacteria are added from the environment, and from this mixture the normal bacterial flora of the child is selected.

Except when successful invasion by external pathogens occurs, the human intestinal flora seems to be a stable, self-regulating system.

Gram-negative bacteria and their endotoxins are ubiquitous. A substantial part of the 10^{14} living bacteria we house in our gastro-intestinal tract is Gram-negative and consists of the families Pseudomonadaceae (e.g. *Pseudomonas aeruginosa),* Enterobacteriaceae (e.g. *Escherichia coli, Klebsiella aerogenes, Proteus mirabilis),* Bacteroidaceae (e.g. *Bacteroides fragilis, Fusobacterium),* Neisseriaceae *(Neisseria catarrhalis, Veillonella parvula).*

A single bacterium contains approximately 10^{-14}g endotoxin (or 1 per cent of the bacterium's own weight), and the endotoxins may be encountered either firmly bound to the mother cell or in a free, dispersed state when they are liberated into the surrounding medium following the death or lysis of the cell. Experimentally, lipopolysaccharide can be split by mild, acid hydrolysis into a lipid A fraction (the lipid A moiety is a complex, chloroform-soluble glycophospholipid), which is apparently responsible for the toxicity, and a polysaccharide, which is responsible for the antigenic specificity. Endotoxins are remarkably resistant to most generally applied detergents and they are extraordinarily heat-resistant (heating to at least 170°C for a period of four hours is required to render them inactive).



A thimbleful of endotoxin or lipopolysaccharide from Gram- negative bacteria. Enough to put a battalion of soldiers to bed with influenza - like symptoms.

Normally we live in mutually beneficial and vital symbiosis with our Gram-negative bacterial flora. This healthy balance is controlled mainly by liver-regulated mechanisms like detoxification, conjugation and clearance of the blood, the measures which prevent absorption of bacteria and endotoxins from the gastro-intestinal tract into the venous system. In addition, a certain tolerance phenomenon to endotoxin will keep our immunological surveillance systems alert to the escape of lipopoly-saccharides into the bloodstream.

When, for one reason or another, these various defense mechanisms are compromised, invading endotoxins may cause a number of often interrelated physiological and pathological effects in the mammalian host, for example, on the temperature-regulating centre in the brain, on the different cell populations in the blood, on the hormonal systems, on the metabolic system and on the immunological surveillance systems, which may ultimately lead to disseminated intravascular coagulation, hypotension, circulatory collapse and, not seldom, death. Despite the introduction of new antibiotics and the increasing sophistication of supportive care, hospitalized patients suffering from Gram-negative bacterial septicaemia continue to have a mortality rate of about 30 per cent, increasing to 70 per cent or even more if shock aggravates the clinical condition, and much recent evidence support the perception that endotoxin is the main cause of many of the pathological features of septic shock.

For many years, the extreme sensitivity of man to the pyrogenic properties of endotoxins has been recognized as the classic effect of lipopolysaccharide. This pyrogenic action is most probably indirect and mediated through an endogenous pyrogen released from polymorph nuclear leukocytes.

Other effects include alterations of the haemapoietic system such as leucopenia and leucocytosis and changes in the coagulation and fibrinolytic mechanisms. Thrombocyto-penia may be seen following the intravenous injection of endotoxin and is quite often encountered in patients with Gram-negative bacterial infections. Endotoxin may lead to increased reactivity to epinephrine resulting in haemorrhagic lesions, may provoke locali-sed and generalised Schwartzman-Sanarelli dermal reactions, and cause placental injury resulting in foetal wastage or premature delivery. Other characteristic effects of endotoxin comprise alterations in the activity of the reticulo-endothelial system and profound changes in metabolism, such as hypoglycaemia, hyperlipidaemia, hypoferraemia and decrease in serum-zinc concentrations. Furthermore, lipopolysaccharide can activate both the classical and alternate pathways of complement, systems of central importance to the mediation of the inflammatory reaction consisting of 11 proteins that constitute about 10 per cent of the globulins in normal, human serum.

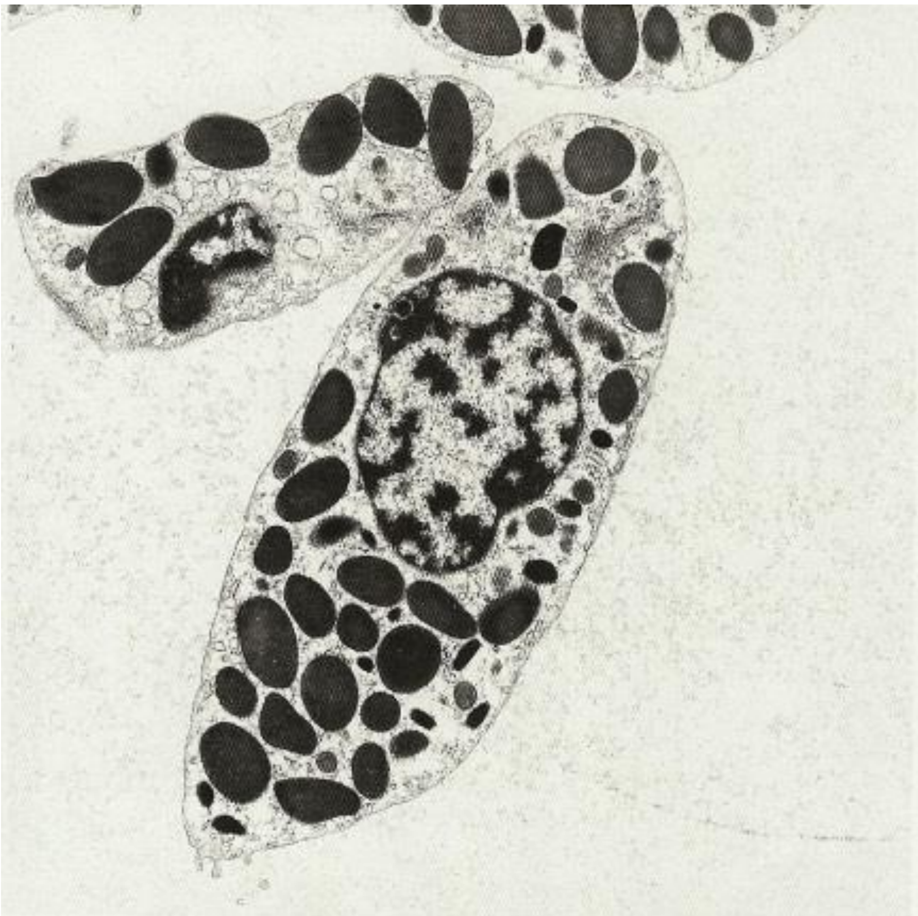
When all this damaging evidence has been presented, it must be stressed, however, that endotoxins are not just foes but also, in some respects, friends of the human and other mammalian families.

Small amounts of LPS can produce a beneficial stimulation of our immunological surveillance, stimulate leucopoiesis in the bone marrow and increase our non-specific resistance to various pathogens. The increased resistance pertains to a variety of bacteria, fungi, parasites and viruses. The increased resistance to viruses is apparently caused by the stimulation by endotoxin of the interferon production, probably mediated by the lipid A moiety of the LPS molecule. Endotoxins may also induce necrosis and regression of malignant tumors, they may act as adjuvances and they can provide protection against the vascular and haemodynamic effects of radiation.

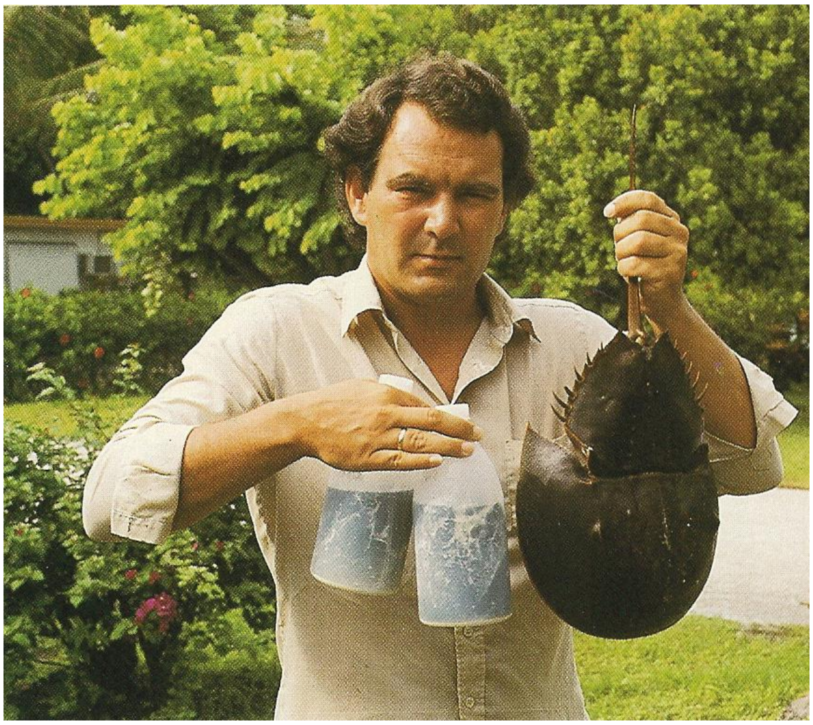
When mentioning small doses in relation to endotoxins, it should be emphasized that as little as 2 nanograms of endotoxin per kilogram of body weight administered intravenously in a healthy adult human being may provoke an increase in body temperature of 1.9°C. Typical reactions to such small doses of endotoxin also include granulocytosis, chills, headache, rigor, muscle pain, loathing of food, nausea and vomiting. Other constant effects include the increase of IgM, IgA and IgG immunoglobulins, factor VIII, cortisol and growth hormone in plasma, and a decrease in serum-iron.

Many biological assays have been employed in the past to detect the presence of these potentially lethal endotoxins. Provoking of fever in rabbits has been the most widely used diagnostic aid, but death and morbidity in other living creatures have also been applied to assess the biological effects of endotoxin, e.g. death in bird embryos, provoking of abortion, the production of skin inflammation and damage to rodent sarcomas have all been used as end-points is such assays.

However, all these assays that are dependent on living animals or living parts of animals are fraught with difficulties and inaccuracies.



The source of the *Limulus* lysate: The amoebocyte of *L. Polyphemus.*



The author with a female. L. polyphemus which has just donated one fifth of its total blood volume. The bottles contain approximately 2 liters of blue blood which have been pooled from several animals. (Plantation Key, Florida)

In 1956, Professor Frederik B. Bang drew our attention for the first time to the fascinating future prospects of a specific and reproducible way of detecting endotoxins, even without having to kill or disable animals.

He revealed the presence of a coagulation principle, extremely sensitive to endotoxin, wholly contained within the amoebocytes of the American horseshoe crab, *Limulus polyphemus.*

This intracellularly derived coagulation system, which had been of vital importance to the horseshoe crabs for hundreds of millions of years as protection against the marine Gram-negative bacteria and their endotoxins, was subsequently extracted from the amoebocytes and developed into an eminently simple and specific in vitro test by Drs. Frederik B. Bang and Jack Levin.

Although other marine invertebrates contain blood cells reactive to endotoxin (e.g. the spiny spidercrab, *Maia squinado),* the horseshoe crabs of all extant species (but especially *L, polyphemus* and *Tachypleus tridentatus)* are unique not only by containing up to 300 ml of easily accessible haemolymph, but also by the extreme sensitivity of the cell extracts, or lysates: *Less than a millionth of a billionth of a gram of endotoxin can be detected in less than one hour.*

The amoebocyte, an elliptical cell measuring about 12 by 18 μm is the only type of circulating cell in the haemocyanin-blue blood, or haemolymph, of *Limulus polyphemus.* The Indo-Pacific species *T. tridentatus* may have two or three types of circulating cells in its circulatory system, although final evidence is still lacking.

The amoebocyte is a nucleated cell and its cytoplasm is densely packed with large refractile granules containing all factors that eventually contribute to the unique blood-clotting system in horseshoe crabs.

When stimulated in *vivo* or *in vitro* by minute quantities of endotoxins (in either a free, dispersed state or a form associated with intact cell-walls of Gram-negative bacteria), these amoebocytes aggregate very quickly while their shapes change markedly and long processes develop that extend from cell to cell in pseudopod-like fashion. These aggregated amoebocytes then degranulate or "lyse", expelling not only the clottable protein (coagulogen) but all other factors required to form the ensuing gel or clot, which quickly immobilizes and engulfs the endotoxins.

Endotoxin cannot enter the intact amoebocyte and cell-free haemolymph does not clot, nor is its presence required for coagulation. Aqueous extracts of washed amoebocytes from either *L. polyphemus* or *T. tridentatus* (the only ingredient contained in the commercially available *Limulus lysates)* undergo coagulation following incubation with endotoxin.

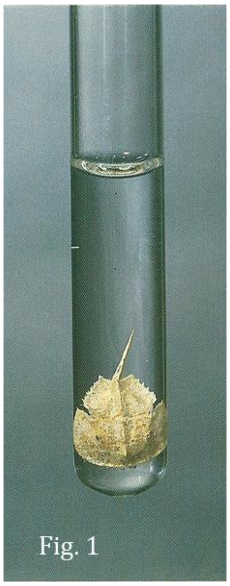
The cellular process and the biochemical mechanism of amoebocyte degranulation and the subsequent gel-formation as the coagulation proceeds to endotoxins have not been fully elucidated, but a tentative and simplified description of the processes is as follows:

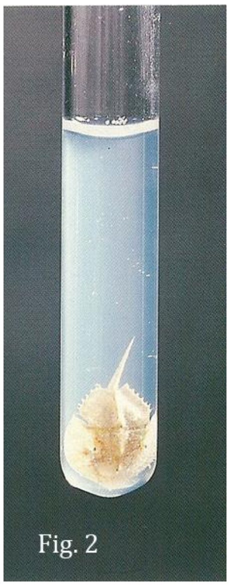
First, a large molecular weight pro-coagulant enzyme (or enzymes) in the lysate is activated by the endotoxin to form an activated clotting enzyme (s). This enzyme (s) then acts on the clottable protein (coagulogen) resulting in limited proteolysis. Polymerization then occurs and a gel is formed, composed of long fibrils which are apparently cross-linked. The coagulation process requires the presence of divalent metal cations, and the resulting proteinaceous gel (coagulin) is insoluble.

The recent identification of an alternative coagulation pathway has been reported by Japanese scientists, and has resulted in the marketing of a modified chromogenic endotoxin assay free of a so-called factor G. This assay seems to be advantageous for the diagnosis of endotoxemia.

The coagulogen of all xiphosurid species has approximately the same molecular weight, namely between 23.000 and 27.000 daltons. The gel that forms is stable for several weeks if kept in a suitable environment. Great care must be taken, however, in handling Limulus test vials during the incubation period, since the gel is extremely sensitive to mechanical disturbance at this point and will not re-form once it has been disrupted.

Today the Limulus test is generally accepted as the most sensitive *in vitro* assay for endotoxin. The test itself is elegantly simple and relatively easy to perform and apart from the persistent demand for pyrogen-free handling the original, basic gel-clot endotoxin test - which is still by far the most widely used - requires no sophisticated equipment:





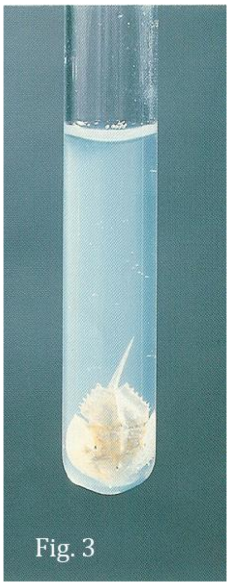


Fig. 1: A baby exuviae of *L. polyphemus* is "witnessing" the performance of a Limulus test. Limulus lysate and water containing endotoxin from *E. coli* bacteria have been thoroughly mixed and the mixture is left absolutely undisturbed.

Fig. 2: After a few minutes the mixture begins almost imperceptibly to change color and density...

Fig. 3: Ten minutes have passed. The endotoxin-concentration in the test tube is one billionth of a gram.

Fig. 4: Complete gelation of the mixture has occurred. From a waterish clear and liquid beginning a milky, bluish jelly-like substance has materialized. Invert the test tube 180° and the "jelly" sticks to the bottom. The Limulus test is positive. A conjuring trick from the Palaeozoic era has been performed successfully.

An equal volume of Limulus lysate and test sample (typically 0.1 ml of each) are mixed in a pyrogen-free test tube, and the mixture is stirred mechanically. The mixture is then incubated for a period of time, usually one hour, at 37°C. At the end of this period the test tube is removed from the incubator and examined for the presence of a gel or clot. If the mixture is unchanged clear and waterish and runs down the wall of the test tube when it is inverted 180°, the sample is not infected or contaminated with Gram-negative bacteria and/or cell-wall endotoxins. If a firm, opaque clot has formed which sticks to the bottom of the test tube when this is inverted, the sample is infected or contaminated with Gram-negative bacteria and/or cell-wall endotoxins. When used in this manner, the gel-clot end-point assay is primarily a pass-fail qualitative test, limited only by the sensitivity of the Limulus lysate employed. By testing a series of 10-fold or 2-fold dilutions of the test sample the gel-clot end-point assay may be used semi quantitatively. If the reciprocal of the greatest dilution of the test sample found to produce a solid gel is multiplied by the labeled sensitivity of the lysate, the approximate level of endotoxin in the test sample can be quantitated. The Limulus test is of the greatest importance in cases where living bacterial cells are absent and free endotoxins, which cannot be stained and "caught" in a normal microscope, are the culprits.

Today four basic methodologies' are available in the commercial Limulus test market: the *gel-clot test* mentioned above, and, secondly, the *turbidometric Limulus test* which is based on the observation that any increase in endotoxin concentration causes a proportional increase in turbidity due to precipitation of coagulogen from the Limulus lysate. The amount of turbidity formed is proportional to the amount of active clotting enzyme and hence to the amount of lipopolysaccharide present in the test sample. This method is in principle an extension of the gel-clot test and after incubation the optical density values can be read on a spectrophotometer following which the endotoxin concentration of the test sample can be extrapolated from a control standard curve. The third methodology is called the *colorimetric test.* It is similar to the turbidometric test except that following incubation the precipitate consisting of turbid (gel-clot) particles is collected by centrifugation and washed free of sample and non-clotted protein. The precipitate is then assayed by the Lowry protein procedure and optical density values of the colored test and control samples are read on a spectrophotometer in the same way as for the turbidometric assay. The fourth method, the *chromogenic substrate test,* is also a quantitative endotoxin test read by spectrophotometry, but it differs from all the preceding assays in that the coagulogen has been completely or partially replaced by a synthetic chromogenic substrate which is covalently bonded to a chromophore (para-nitroanilide). The cleaving of the chromophore by the activated proclotting enzyme produces a color that is proportional to the concentration of endotoxin present in the reaction mixture. The colored samples can be read in a spectrophotometer, as mentioned above.

Adult *T. tridentatus* collected in a tide pool immediately prior to partial bleeding procedures in the Beihai Lysate Factory. Even in temporary captivity the male will attach itself to the female, or literally queue up as in this picture.

Several additional methods have been developed, mostly as more or less practical modifications of the four basic principles already mentioned.

The most sophisticated test principle available today is a combination of the gel-clot method and the rocket immunoelectrophoretic method which measures the loss of the antigenicity of coagulogen when it is split by the endotoxin-activated enzyme.

This test principle, which is based upon anti-coagulogen antibodies, requires a fairly high level of skill and is quite time consuming to perform, but it may become of significant value for diagnostic purposes in clinical medicine.

In the near future we may expect a new and very promising development within the I Limulus test technology, namely an enzyme-linked immunosorbent assay employing monoclonal antibodies against lysate-coagulogen from both *L. polyphemus* and *T. tridentatus.* These two immunological Limulus test innovations have both been designed at the University Clinic for Infectious Diseases, Department M, Rigshospitalet, Copenhagen, and the latter technique in particular could conceivably spearhead the universal acceptance of the Limulus test for clinical diagnostic use.

Although the Limulus assay is uncomplicated to perform when it is applied to simple fluids, we have indeed encountered problems in connection with complex fluids such as blood. The enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies may be the solution.

If at the same time all medical doctors could be convinced that the production of fever is probably the least important effect of endotoxin, and that they should think about "endotoxicity" instead of "pyrogenicity" when dealing with Gram-negative bacterial infections, a new era would begin.

Chinese horseshoe crabs kept in sea-farms until they have donated blood for the cell harvest in Xiamen, China.

A basket full of adult *T. tridentatus* immediately prior to the bleeding procedures at the Xiamen Amebocyte Lysate Factory.

A Scientific Revolution

The production of the first commercially available Limulus Amoebocyte Lysate (LAL) can be traced back to Woods Hole in the 1970's, when Stanley Watson and James Sullivan founded Associates of Cape Cod, Inc.

Prior to that seminal event, I, at the University Clinic for Infectious Diseases, Rigshospitalet, Copenhagen, and, I suspect, many other needy laboratories around the world, got a number of vials of highly sensitive LAL from Watson's own laboratory free of charge, but, eventually, we had to face, of course, the harsh realities of this world.

Today, there are eight companies licensed by the U.S. Food and Drug Administration (FDA) to make LAL. Of these eight, Associates of Cape Cod received the first license in 1977, and this internationally well-reputed company continues to deliver one of the best lysates produced in the United States. The lysate production is seasonal, beginning in the spring with the appearance of horseshoe crabs in coastal areas preparing themselves for the spawning season. The basic procedures for the production of lysate are the same whether the source is *Limulus polyphemus* or any of the Indo-Pacific species:

Whole blood (haemocyanin-blue haemolymph and granula-dense amoebocytes) is withdrawn via heart puncture using a large gauge needle into centrifuge tubes and centrifuged immmediately at low speed in order to prevent premature disruption of the extremely fragile cells. When the intact amoebocytes have been sedimented, the cell-free haemolymph is decanted and deep-frozen for later use in other connections (or, by some produces the haemolymph is simply discarded). The sedimented lump of amoebocytes is then homogenized mechanically, following which the homogenate is transferred to centrifuge and diluted with depyrogenated water (usually 1:3).

"Ballooning" gills in a male *T. tridentatus* from Xiamen, China.

Sometimes even a horseshoe crab falls ill, and by the slightest sign of disease it should certainly not be bled for the production of amoebocyte lysate.

Moribund and recently dead horseshoe crabs are frequently found with thick layers of bacteria on the surface of their gill-books, and in some the gill leaflets may be dilated or "ballooned" as in this illustration. Erosion of the carapace and bran-cilia' appendages may be initiated by slight physical injury or by ectocommensals, and once the armour is penetrated the erosion may quickly be further aggravated by the activity of chitinoclastic marine bacteria and fungi.

After centrifugation at high speed, the cell debris has been sedimented and the clotting factors, liberated from the disrupted amoebocytes, float in the supernatant. Finally this supernatant, which is in fact the Limulus lysate, is transferred to pyrogen-free vials ready for use. The amoebocyte lysate is stable at room temperature for several weeks (but it certainly should not be left on the window sill on a sunny afternoon) and at -20°C it can be stored for years with no decrease in sensitivity.

The Limulus test systems can detect as little as 10^{-15}g lipopolysaccharide in a sample as small as 0.1ml or even smaller. The endotoxin positive test is easy to define, it is reproducible and specific, although the specificity to endotoxin has been debated intensely at times.

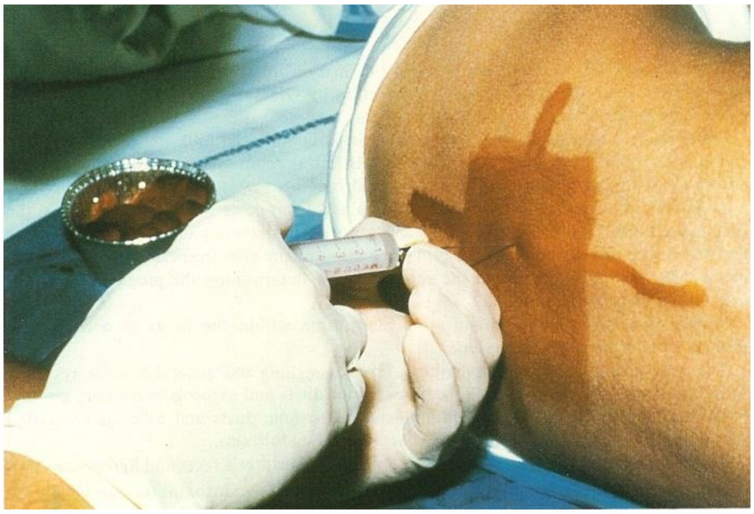
It has been demonstrated that the cell wall peptidoglycans from certain Gram-positive bacteria, like staphylococci and streptococci can elicit gelation of the lysate, but, in contrast to living Gram-negative bacteria, viable Gram-positive micro-organisms do not produce a positive Limulus test - this is achieved exclusively with peptidoglycans isolated from the cell. And it should be stressed that peptidoglycan was 1.000 to 400.000 times less reactive than endotoxin from *E. coli.* Similarly, certain streptococcal exotoxins have produced a positive gel-clot Limulus test, but like the Gram-positive peptidoglycan these exotoxins are several thousand times less active on a weight/volume basis than endotoxins.

In all normal and realistic connections the Limulus test *is* functionally specific for endotoxin, and, provided that the strict demands for pyrogen-free utensils are fulfilled, the performing of the test is uncomplicated and safe.

Since its introduction, the Limulus test has been used successfully for the detection and measurement of LPS in a variety of scientific disciplines, although the major use of the test today is for the pyrogen testing of pharmaceutical products, due to the fact that the test is simple and much less expensive that the rather unspecific rabbit test.

In biomedical science the Limulus test has been applied to examine blood from patients suspected of Gram-negative sepsis and to study experimental endotoxaemia and shock in laboratory animals.

Applied to determine endotoxins in cerebrospinal fluid (CSF), the Limulus test, in one study, was positive in initial specimens of CSF from all eighty-six patients with Gram-negative meningitis, and uniformly negative with the remaining 146 patients with a variety of infectious and non-infectious conditions. Endotoxin concentrations in initial specimens of CSF from patients with Gram-negative bacterial meningitis ranged from 4 to 2.000 nanogram per ml. In comparative evaluation of the Limulus test and the traditionally employed direct Gram-stain for the detection of significant bacteriuria, the Limulus test was both more sensitive and specific than the Gram-stain procedure and much less susceptible to errors of interpretation than methods involving microscopy.



Cerebrospinal fluid can be tested directly and immediately with the Limulus test, thus diagnosing a Gram-negative meningitis in less than one hour.

The Limulus assay revealed the presence of bacteriophages and endotoxin in four live virus vaccines (poliomyelitis, measles, mumps and rubella) and in commercially prepared calf sera. The equivalent of 1.000 nanogram endotoxin per ml was present in some of the vaccines and bovine sera, and the Limulus test was recommended by this study group as a sensitive and specific indicator of any pre-existing Gram-negative bacterial contamination of animal sera or other constituents used in the cell-culture phase of virus vaccine production.

In fact, it was the Limulus test and not the rabbit test which, in 1976 in the United States, demonstrated that certain lots of influenza vaccine were heavily contaminated with endotoxin, and as a direct consequence of that discovery FDA regulations now require a test for endotoxin content in addition to a pyrogen test prior to the release of influenza vaccine. This influenza vaccine incident elicited the first official FDA recognition of LAL for use with a drug product, and with FDA acceptance the application of the Limulus test increased dramatically.

The Limulus test in early Chinese editions applying cell-lysates from *T. tridentatus. Chinese ly*sates are now entering the international market because of their high quality and the large populations of *T. tridentatus* in main areas.

In 1973 it was hypothesized that bacterial endotoxin contamination of radiopharmaceuticals caused the aseptic meningitis (characterized by neck stiffness and severe headache) that was observed in patients following the intrathecal administration of radiopharmaceuticals for cisternography.

The basis for this hypothesis was derived from accumulated experience with radioionated serum albumin. When samples from identical lots of these radiopharmaceuticals were available for testing after clinical use, it was demonstrated that the lots used in patients who developed aseptic meningitis were all contaminated with endotoxin according to evidence presented by the Limulus assay, while *all* lots were pyrogen-negative when tested in rabbits using the U.S. Pharmacopeia procedures.

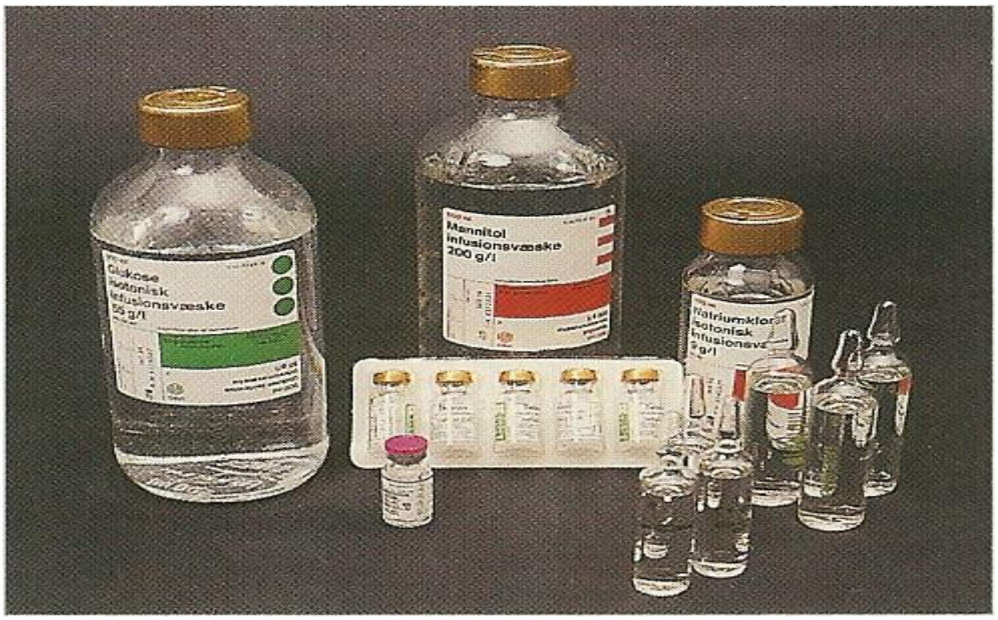
In 1983, the use of the Limulus Amoebocyte Lysate test in connection with short-lived radiopharmaceuticals was made an official part of the U.S. Pharmacopoeia procedures.

Quantitative studies have shown aseptic meningitis to be associated with endotoxin levels of 1 to 100 nanogram per injection of cisternographic drugs.

Endotoxin is also suspect in certain cancer chemo-therapeutic agents that have a high incidence of adverse effects. For example, fever, nausea, vomiting and haematological changes are prominent adverse reactions to L-Asparaginase therapy that are due in part to endotoxin contamination. In a study performed in 1979, seven out of a total of nine antitumor agent preparations examined were positive in the Limulus test.

Furthermore, the LAL test has been applied to evaluate bacterial contamination in blood processing, to detect endotoxin in fluids associated with haemodialysis procedures and, since chorio-amnionitis can be caused by endotoxin-producing bacteria, the Limulus assay has also been used to detect endotoxin in human amniotic fluid.

One very recent (1987) clinical application for the LAL reagent is for the diagnosis of gonorrhea, which is caused by the Gram-negative bacterium Neisseria gonorrhea possesing an endotoxin that is extremely reactive with the Limulus test. The commercial name for this latest LAL-development is Gonoscreen and it is described as a test for in-office presumptive diagnosis of male gonococcal urethritis by it manufacturer, Mallinckrodt Diagnostic Products Division.



The pharmaceutical industry almost immediately recognized the importance of the Limulus test for the quality control of their products.

The Limulus test is useful for the detection of bacterially contaminated fish, meat (including frozen goods), milk and other dairy products. In a study in Denmark published in 1980, we were able to conclude that the Limulus test is a rapid, reliable and valuable method of ascertaining the hygienic quality of market milk and several other dairy products, determining the accumulation of infection by Gram-negative bacteria during milk production and processing to the final products.

Other studies have suggested that endotoxin in commercial milk samples and the increase in endotoxin concentration when milk is left at room temperature may be contributory factors to the Sudden Infant Death Syndrome.

In several studies of milk from normal cows and from cows with clinical mastitis, the LAL assay proved to be highly specific and sensitive in detecting endotoxins, suggesting that the Limulus test could also be applied to monitor the effectiveness of (specific) antibiotic treatment and perhaps aid the veterinarian in determining the prognosis of the Gram-negative mastitis of cows.

Finally, the LAL test is gaining rapid recognition within the fields of occupational medicine and environmental hygiene.

Danish dairy products, world famous for their high quality, are routinely checked for endotoxin by means of the Limulus test.

The recognition of pulmonary injury, fever, coughing and general malaise resulting from the inhalation of microbially contaminated dusts and aerosols is growing rapidly. Foremost among the microbial contaminants of organic dusts and aerosols associated with these symptoms are Gram-negative bacteria and endotoxins.

Earlier reports about mill fever, hemp fever, Bible printer's fever and *Befeuchterungsanlage Fieber* demonstrate that Gram-negative bacteria and endotoxins (or pyrogens) have been associated with occupational medicine as far back as 1784.

More recent studies show that the diseases ascribed to the inhalation of microbially contaminated dusts in the textile industry often appears in small epidemics, the main symptoms being strikingly similar to the well-known effects of endotoxin.

It is generally recognised that the injection into cerebrospinal fluid of endotoxin is highly toxic while the inhalation of LPS resembles the effects following intravenous injection. Tests have shown that workers exposed to waste water aerosols at sewage treatment plants may experience endotoxin levels above 100 microgram per day, a dose which is above that required to produce medical symptoms. Even processed waste water sometimes used for irrigation or cleaning purposes may contain around 5 microgram per ml.

Metal-working fluids normally contain from 1 million to 60 million microbes per ml under industrial conditions, and counts as high as 700 million bacteria per ml have been reported. The most important organisms capable of growing in industrial oil emulsions, both mineral-oil and synthetic lubricants, are Gram-negative bacterial species. Metal-cutting operations often result in the production of a stable mist, and this aerosol can spread contamination on walls, floors, equipment and, of course, in the human operators. Once more, the Limulus assay clearly indicated the health hazards of oil droplets that act as carriers for a wide range of bacteria and lipopolysaccharide.

Small wonder, indeed, that the rabbit has been overtaken and left astern by our phenomenal living fossil when one considers the tremendous contributions this diverse and specific Palaeozoic principle has made - directly and indirectly - towards the improvement of the quality of human life.

EPILOGUE

Horseshoe Crabs Today ... And Tomorrow?

Evolving with (and as) the Tethys Sea in Precambrian times, water was accumulated from the interior of the Earth to build our hydrosphere. It is generally assumed that most, if not all, water appeared within a relatively short period of time, and that little - if any - new water is being generated now.

Water, the *sine qua none* of life, has been in proper use for hundreds of millions of years on this planet, but with the advent of *Homo scientificus* in ever increasing numbers, it took us about 100 hectic years to create the present dangerous level of pollution and general overburdening of our natural waters with non - degradable or slowly degradable waste.

Although water becomes dirty with proper use, it must certainly be dirtied with care, and respect for the fact that ultimately water is a non-renewable resource in the sense that it may become an impossible task to clean it sufficiently to support life.

The world ocean, holding 1.370 million cubic kilometres of the total amount of water on our planet and covering 71 % of the planet's surface, has an enormous capacity to assimilate simple and naturally occurring waste, but when it comes to toxic materials (e.g. heavy metals), highly persistent chemicals (for instance chlorinated hydrocarbons and their decay products), dissolved salts (e.g. the sodium ion) or radioactive materials, there is no assimilative capacity any more, although a certain dilution may be expected.

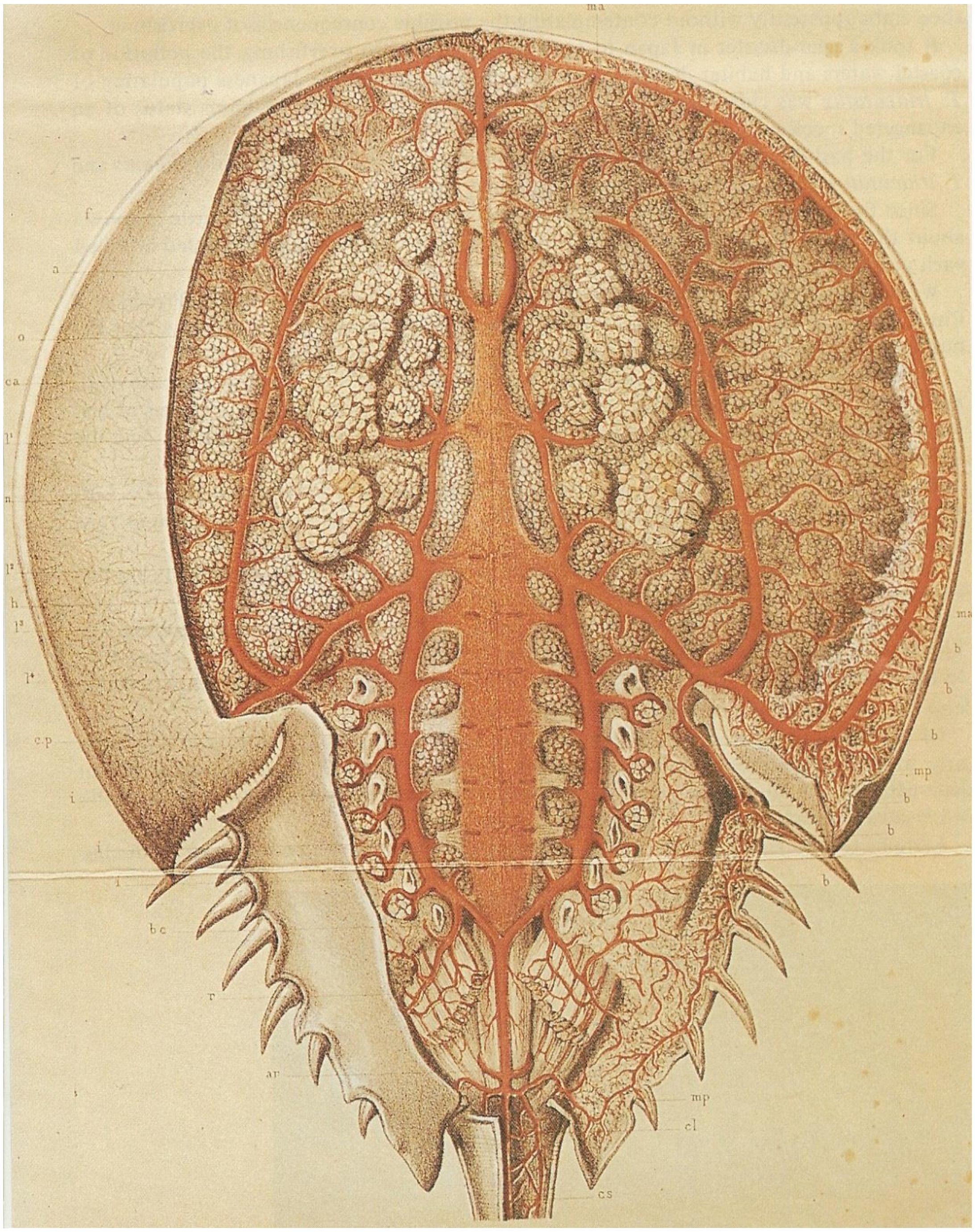
These kinds of waste are quite often deposited in the nonproductive areas of the sea with an erroneous feeling of safety. Erroneous, because they may reappear in the productive areas at some future time.

Even though the open sea, which represents about 90% of the total surface area of the world ocean, is a virtual desert that contributes a relatively small fraction of the world's fish catch, the remaining 10% is probably going to be of vital importance to mankind in the next century, not only to supplement our dwindling terrestrial resources, but also as a treasure chest of flora and fauna, from which we will gain important scientific knowledge.

Until very recently, man took as much as possible from the sea without trying to support, protect, manage or increase the ocean's live resources.

And with few exceptions (some of them already mentioned in this book) scientists and businessmen helped, to a lesser extent, by fishermen have for years exploited the horseshoe crabs apparently without contemplating the possible consequences of overfishing.

It took a near-disaster in Japan to open our eyes: Due to overfishing, the pollution of coastal waters and habitat destruction (e.g. land reclamation) the Japanese population of *T tridentatus* was close to annihilation and is now reduced to the dubious status of an endangered species.



The circulatory system of *Limulus polyphemus - a* beautiful manuscript drawing - but the blood *is blue.*

For the past 10 years a large number of horseshoe crabs (notably *L. polyphemus* and *T tridentatus)* have been used in biomedical research.

Since the Atlantic states do not regulate the taking of horseshoe crabs, little is known about the present population of *L. polyphemus* and the number of animals bled or killed each year in the U.S.A.

We do know, however, that 50.000 *T. tridentatus* were bled in the Fujian Province of China over a 7-year period from 1978 to 1984, and it is reasonable to assume that the number is several times higher for *L. polyphemus* in the United States.

In America today, concerned scientists are expressing fear that the increased use of *L. polyphemus* as eel and conch bait combined with the harvesting of large numbers of animals for medical purposes may constitute a threat to the survival of the species. And the naked figures speak for themselves:

In a 1977 shoreline survey of the spawning horseshoe crabs in Delaware Bay, Dr. Carl Shuster concluded that the peak population at the time of full moon tides in June numbered about 220.000 males and 51.000 females.

In 1856, however, more than 1.2 million horseshoe crabs were caught along just a few kilometres of the Cape May beach in Delaware Bay.

Delaware Bay, Cape May beach, 1856.

It should be kept in mind that horseshoe crabs are slow growing animals requiring 9 to 11 years to attain sexual maturity. If they are heavily harvested, it may take a long time to repopulate an area. And it should also be kept in mind that bled animals experience at least a 10% higher mortality rate than unbled controls.

Of the three acknowledged Chinese species of horseshoe crabs *T. tridentatus* is particularly abundant. Lysates produced from the amoebocytes of *T. tridentatus* have been demonstrated on numerous occasions to be of equal sensitivity and high quality to the cell lysates produced from *L. polyphemus.*

Limulus test users all over the world, and especially the American companies producing and selling the Limulus lysate on the international market, should acknowledge the introduction of the Chinese Limulus lysate produced from *T. tridentatus,* as well as the excellent Japanese test kits, and consider these commercial initiatives a welcome opportunity to diminish the pressure on the American horseshoe crab populations, rather than fight the newcomers in the traditional way of the business community.

The Limulus Amoebocyte Lysate test for the detection of Gram-negative bacterial endotoxin has become indispensable to biomedical science, for the protection of our environment and to the pharmaceutical (and many other) industries, and it is moving into new, important fields of application every week.

Thus, there can only be one reasonable conclusion to "The Secret in the Blue Blood":

Science and industry must retain this unique diagnostic weapon, and planet Earth must retain her most distinguished inhabitant.

A simple principle of genius invented by the *Xiphosura,* discovered by Professor *Frederik B. Bang* and developed by him and *Dr. Jack Levin.*

Performed today all over the world and the subject of intense research in order to create even more sophisticated testing procedures.

The Secret in the Blue Blood has been revealed, but in the process new secrets have emerged.

The eternal quest for truth and understanding *is* eternal…

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