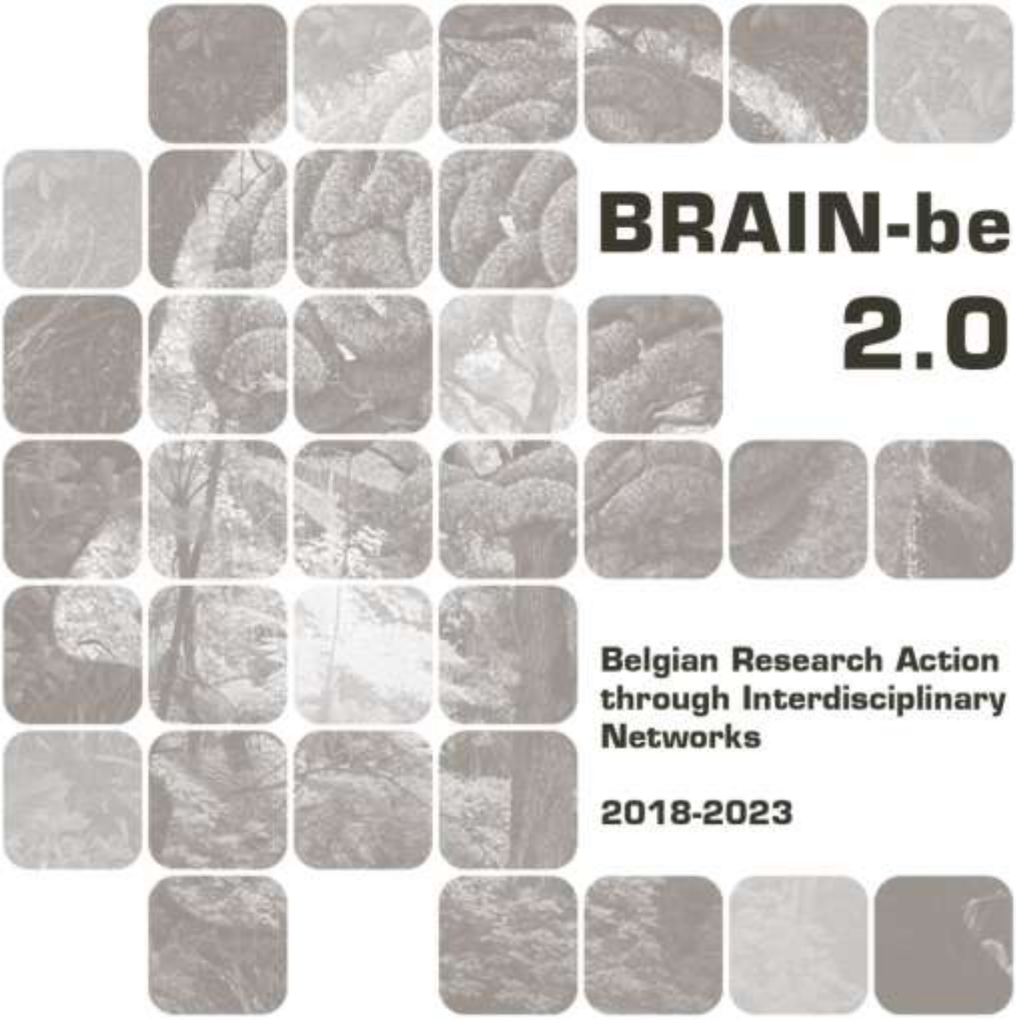

PARADI^{2}S

PARAsitic DIversity, vectors, hosts, and transfers in Early Cretaceous DInoSaur-associated vertebrates

NINON ROBIN (IRSNB) – KEVIN REY (VUB) – PASCAL GODEFROIT (IRSNB)

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Belgian Research Action
through Interdisciplinary
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NETWORK PROJECT

PARADI^{2}S

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ABSTRACT

Context

Parasitic animals are known to have evolved with the acquisition of a complex life cycle passing through egg, larvae, multiple cysts (i.e., resistance stages), and usually a brief adult stage. The segmentation of parasites' life cycle has appeared along with the acquisition of several – and very different – hosts over one individual lifespan. We know that significant switches in hosts are thought to happen along with (1) a biotic expansion of the parasites and (2) ecological perturbations on the side of the hosts, the latter applying largely to most terrestrial vertebrates in the context of rapid climate change and drastic reduction of wild territories in profit of livestock exploitations. We ignore though if parasite transfers between vertebrates always occurred this way ? Early on, could different animals have played specific roles in disease transfers as vectors and which were their animal hosts ? To tackle these main questions, the 4-years PARADI^{2}S project offers to study (1) mineralized 150-100 million years old faeces of vertebrates for their content in internal parasites, in order to document parasitic changes over time; and (2) ancient ectoparasitic animals preserved in amber to understand how they changed in hosts over time.

State of the art and objectives

The PARADI2S project aimed to:

- Unravel the chemical signal contained in mineralized fossil stools.
- Examine collections of exceptionally preserved plumage/furs of Early Cretaceous dinosaurs/mammals.
- Analyze the endoparasitic content of several hundreds of vertebrate coprolites.
- Infer direct vertebrate host switch by main lineages of parasites.

Results

- WP1. We studied about 600 fossil feces of successive geological ages and characterized the vertebrate
- WP2. We assessed the elemental chemistry of fossil feces and showed it reflects diagenesis
- WP3. Assess the isotopic signal of fossil feces and showed it can be a proxy for paleoenvironmental reconstructions
- WP4. We identify 30 fossil endoparasitic inclusions and designed a project to distinguish them from fossil pollens
- WP5. We described new cases of Early Cretaceous ectoparasitic arthropods and set a benchmark for the recognition of fossil blood in amber and then of hematophagous insects.
- WP6. We discovered and unveiled the paleobiodiversity of a co-eval fossil site of that of Bernissart, Belgium, which is worldwide famous for providing among the first described dinosaurs.

Conclusions

PARADI^{2}S project allowed the following conclusions:

- The recognition of fossil parasites in mineralized faeces implies more comparative taphonomy studies to be properly done
- It is though well-preserved calcium-phosphate faeces and could inform on host-parasite transfers
- Amber can preserve ancient molecules indicative of ancient ecologies
- Fossil faeces could be used as paleoenvironmental proxy, in place or complement of skeletal remains

It aligns with RBINS's strategic goals for 2020–2025, enhancing our understanding of how parasites and micro-organisms evolve through time depending on their hosts environments.

Keywords

Parasitism, fossil feces, amber, terrestrial vertebrate assemblages, elemental chemistry, isotopic signal, mites, blood-sucking insects, helminths identification.

1. CONTEXT

Infectious diseases known as zoonoses are transmissible between animals and humans. When the animals act as reservoirs for the pathogen and humans accidentally get infected, the infection is defined as an anthropozoonosis. Zoonotic diseases correspond to cases where pathogens have been able at some point to switch from their usual host to a new one onto which its pathogenicity significantly increases. These diseases correspond to a range of transmitted pathogens. They can be viral (like COVID-19), bacterial, fungal, or even parasitic when they involve animals. Parasitic animals are known to have evolved with the acquisition of a complex life cycle passing through egg, larvae, multiple cysts (i.e., resistance stages), and usually a brief adult stage. The segmentation of parasites' life cycle has appeared along with the acquisition of several – and very different – hosts over one individual lifespan. These multiple hosts testify of the pathogen transmission from one host to another over its evolution. We know that significant switches in hosts are thought to happen along with (1) a biotic expansion of the parasites and (2) ecological perturbations on the side of the hosts, the latter applying largely to most terrestrial vertebrates in the context of rapid climate change and drastic reduction of wild territories in profit of livestock exploitations. However, many key questions remain: 1) Have parasite transfers between vertebrates always occurred this way? 2) Were they necessarily driven by these two main suggested dynamics? 3) Early on, could different animals have played specific roles in disease transfers as vectors and which were their actual hosts?

2. STATE OF THE ART AND OBJECTIVES

Investigating the process of parasites transfers from one host to another requires either to have access to parasite/hosts history over a long period of time or to be experimentally reproduced. It is not ethically defendable to artificially infect random vertebrate lineage, so far parasite-free, with novel parasites. Fossils are elements possibly tackling the dynamics of these parasite transfers. The first half of the Cretaceous period (-145-100 Ma) has undergone a progressive global warming characterized by a steady atmospheric CO_2 increase, which started at the end of the Jurassic and reached its thermal maximum at the Turonian about 95 Ma ago. At that time, terrestrial vertebrate communities underwent a main radiation event referred as the **Cretaceous Terrestrial Revolution** that is associated to the diversification of mammals and avian dinosaurs (including birds). Along with their body remains (skin, feathers, hair) preserved in Cretaceous sediments and in amber, the stools of these million years old animals fossilize, concentrating samples of their original habitat as digested food. The remains found in these fossilized feces (called "coprolites") are known to show an exceptional state of preservation including plants with their cuticles, animal muscle fibers, embryos, but also eggs and cysts of intestinal parasites. The access to numerous (1) coprolites from different dinosaur-bearing terrestrial environments of the Early Cretaceous and (2) host-ectoparasites fossil remains constituted a great opportunity for the collaboration of paleontologists, geochemists and veterinary parasitologists and to address the following broad questions:

- ***Apart from diet, how do million years old fossil feces inform on ancient (a) animal producers, (b) parasitic loads, (c) paleoenvironments?***
- ***Which parasite transfers have occurred among terrestrial vertebrates over 30 Ma of progressive warming and do these correlates with contexts of radiation or stress pressures?***
- ***What is the succession of main vertebrate hosts for blood-sucking arthropods, and could they have been the vectors of significant diseases waves among ancient vertebrates?***

To tackle these main questions, the 4-years PARADI²S project offered:

(1) To test for, and to potentially unravel, if – as it was recently suggested by geologists and paleontologists – the chemistry of fossil stools could serve as a proxy to the recognition of (a) animal producer categories, (b) burial conditions, and/or (c) paleoenvironmental conditions. These questions were assessed using elemental and isotopic spectrometries (Laser-induced breakdown, micro-X-ray fluorescence, and LA-ICPM spectrometries).

(2) To examine collections of exceptionally preserved plumage/furs of Early Cretaceous dinosaurs/mammals from the China (Jehol Biota) as well as famous amber (fossilized plant resin) record (Lebanon and France) for the preservation of in situ or ex situ external parasites (insects, arachnids). In parallel, the material of engorged insects (mostly different infraorders of flies) recovered from these deposits would be analyzed for the chemical signature of their engorgements, confirming - or not - the emergence of hematophagous lifestyles in these arthropod lineages in the Early Cretaceous.

(3) To analyze the endoparasitic content of several hundreds of vertebrate coprolites coming from four dinosaurs-associated terrestrial environments from the Western Tethys: Angeac-Charente, France; Bernissart, Belgium; Mount-Lebanon; Teruel, Spain) to constrain the distribution of parasites among vertebrates lineages (sharks, actinopterygians, lungfishes, coaelacanths, lizards, crocodilians, dinosaurs, turtles, mammals). A set of cutting-edge high-resolutions imaging methods were added to classic thin section microscopy to recover ancient egg/cysts within mineralized stools like micro-CT, in addition to a newly developed protocol of extraction. The stool producer's identity, and the fossilization history of the fossil stools

(4) To infer direct vertebrate host switch by main lineages of parasites (oxyurid/ascarid nematodes, trematode/cestode flatworms identified by morphotypes) over the Late Mesozoic relative to host lineage ecological fits. The identified ectoparasitic relationships (mites, fleas, mosquitoes-relatives) would then be discussed as regard to hosts paleoecosystems (temperatures, environment) and diversification/extinction trends. Both endo-/ectoparasitic inferences are compared with trends observed in rapid zoonotic infections/epidemic dynamics in wilderness or livestock farms.

3. METHODOLOGY

3.1. Fossil material

Although as old as life itself, parasites and their different developmental stages remain particularly rare in the fossil record. Indeed, parasites are mostly soft-bodied organisms and occupy niches that do not frequently fossilize, i.e. digestive tracts, plumage, and fur. Consequently, the fossil record of parasites is restricted to contexts of exceptional preservation (*i.e.* to deposits that called *Konservat-Lagerstätten*). Contrary to those of other geological ages, Early Cretaceous parasites are actually likely to be found in the three fossil contexts of exceptional preservation targeted in the present project:

a. Fossil faeces (coprolites). Fossil stools concentrate evidence for ancient endoparasitism in the paleontological and archeological records. While the amber record of parasites stays heavily biased towards arthropods and aerial/terrestrial lineages, the endoparasitic fossil record preserved in coprolites ranges from coccidia and other protozoans, parasitic fungi and plant remains, to helminths (including acanthocephalans, nematods, and various groups of parasitic flatworms). The Angeac-Charente (France) collection of vertebrate coprolites (140–135 My-old) is available for study at the Museum of Angouleme, France; the historical Bernissart coprolites (125–120 My-old) are available for study at RBINS; and the — intermediate in age — coprolites from the West Lebanon Mount sites (Snyya, Jdaydet Bkassine) (130–125 My-old) are available at the Lebanese University, Beirut, Lebanon. We plan to collect additional specimens from these localities to document the animal producers of these fossil feces; an excavation fieldwork will be organized in collaboration with Pr. Dany Azar (*Lebanese University*) and his team. Collected specimens will be housed in the *Museum of Natural History* of *the Faculy of Sciences II* of the *Lebanese University*, Fanar. Finally, Triassic coprolites of Southern-Poland (230–200 My-old) providing for an earlier standard of vertebrates endoparasites, are also available for study at the *Institute of Paleobiology* of the *Polish Academy of Sciences*, Warsaw, with the support of the local curators.

b. Fossil ambers. Ectoparasites found in direct associations with dinosaur feathers were punctually reported yet in Cretaceous ambers (Myanmar amber), involving common ticks and more interesting totally novel and curious insects, described as mesophtirids. Endoparasitic animals (e.g. nematodes) are known to occur in fossil ambers, sometimes even escaping their actual insect hosts as in Myanmar amber instances. For essential ethical reasons, this project follows the recommendations of the *Society of Vertebrate Paleontology*, avoiding the controversial Late Cretaceous amber from Myanmar. It will focus instead on — even older — fossil ambers from the Early Cretaceous of Lebanon (lower Barremian). Lebanese ambers are among the oldest ambers on Earth and are known to yield a massive number of invertebrate inclusions, along with the co-eval Isle of Wight Wealden amber (Wessex Formation, UK), which is much less productive in fossil remains. Lebanese amber gathers a large diversity of blood-sucking flies-related insects (Ceratopogonidae, stem-Chironomidae, Psychodidae, Raghionidae, 1150 specimens) and mites (275 specimens). The oldest representatives of hematophagous psychodid flies, which are now the fly vectors of several parasitic diseases including the *Leichmaniasis*, were identified from these Lebanon Early Cretaceous ambers. These arthropods collections are available for study at the Dept of Biology, Faculty of Sciences II at the Lebanese University, Fanar.

c. Volcano-sedimentary deposits of vertebrates (ex: Jehol biota, Liaoning, China). Exceptionally preserved fossils of blood-sucking insects (fleas and relatives) have been described from this famous Early Cretaceous Konservat-Lagerstätte from North-Eastern China. Up to now, the ectoparasites from those deposits were only described isolated from their hosts. In the present project, we examine the large vertebrate collections from the Barremian-Aptian Jehol Biota of Liaoning Province housed in the *Paleontological Museum of Liaoning* (Shenyang, Liaoning Province) and in the *Paleontological Museum of Jilin University*, which contains hundreds of dinosaur, bird and mammal specimens with exquisitely preserved plumage and fur. Official bilateral agreements are already signed between those institutions and RBINS, leading to publications in top-ranked journals. Careful examination of the integumentary structures (feathers/hair/skin) of those vertebrates is likely to reveal the presence of ectoparasites and/or damages caused by those parasites.

3.2. Analytical approach

Insights into the composition of parasite faunas in relatively recent archaeological material are usually obtained using immunological techniques ^{or} ancient DNA sequencings. Unfortunately, the timing of decay in DNA and proteins exclude such applications to older material. Consequently, only direct evidence of associated fossils can unravel parasitic communities in Cretaceous vertebrates.

The rarity and exceptional character of specimens discovered in amber and in the volcano-sedimentary deposits from the Jehol Biota (Liaoning, China) also limit their possible analyses to non-destructive methods. Recent developments in imaging technology fortunately have direct applications to the study of parasitism in natural history collection specimens. Recently, **CT technologies with high-resolution imaging capabilities** (micro- and nano-CT, PPC-SRµCT, FIB-SEM tomography and µXRF) have been developed and are now accessible for researchers. Some of those technologies were briefly experimented but have not yet been deployed for parasite detection or identification in natural history collection specimens[18]. For the first time in the scope of the pioneering PARADI^{2}S project, they are applied on parasites preserved in amber and coprolites.

Contrary to amber and "hairy" vertebrates, coprolites consist of much more abundant material that can easily be sampled for destructive analyses. In the present project, we follow the proposition of a multi-proxy protocol applied for the analysis of Quaternary coprolites to study them. First, the Cretaceous feces are µCT-scanned *in toto*, then sub-sampled for thin sections (light microscopy, petrography, LIBS, µXRF), then nano-CT/synchrotron tomography/FIB-SEM and studied by dissolutions. Fossil endo-/ectoparasites are identified by direct to comparison to modern eggs/cysts/adults collected from livestocks and wild hosts. Vertebrate hosts/endoparasites patterns are mapped out over Triassic, Cretaceous and Modern data for inference of potential transfers over time. These likely transfers confronted to their associated ecosystems (climate/genetic speciation) inform on triggering contexts of endoparasitic zoonotic diseases among vertebrates over time.

Besides their parasitic loads, which are studied using light/SEM microscopy and high-resolution CT imaging, the feces also contain a complex mixture of chemical compounds, including substances from the diet and digestive processes. Coprolites consequently represent remarkable snapshots of the lives of extinct animals, informing on how they interacted with their environment, what they ate, and potentially why they became extinct. In the context of this "parasites" project, it is particularly interesting to compare the discovered endoparasites of vertebrates in several coeval levels and to correlate them with environmental and climatic data. Significant concentrations of calcium and phosphorus in bone and flesh often favor the preservation of carnivore feces by providing autochthonous sources of constituents that result in calcium phosphates mineralization. They concentrate organic phosphates from their environment that can be sampled. The **stable isotope analysis of coprolites** of carnivorous vertebrates is tested here to determine if it constitutes a new relevant proxy for estimating the evolution of palaeotemperatures in Lower Cretaceous terrestrial environments. Their oxygen composition ($\delta^{18}O_p$) profile can be compared to other paleoenvironmental archives of the same age and condition as well as with present-day seasonal records of $\delta^{18}O$ of surface waters ($\delta^{18}O_{mw)}$ values provided by climate stations operated by the International Atomic Energy Agency-World Meteorological Organization (IAEA-WMO) and those comparisons can be discussed in terms of seasonal variations of air temperature, amounts of precipitation and continentality.

To correctly carry out stable isotope analyses, it is essential to firstly understand adequately the **burial/mineralization/diagenetic processes** leading to the exceptional preservations of the coprolites and their content. It is, for example, fundamental to target primary phosphates that recorded — directly or indirectly (via digested preys) — the isotope composition of surface waters, and not secondary phosphates, which result from burial mineralization processes. Understanding those burial processes also requires cutting-edge technologies, such as LIBS, luminescence and µXRF mapping to disentangle possible diagenetic effects, which are absent or minimal in more recent bio archaeological contexts.

4. SCIENTIFIC RESULTS AND RECOMMENDATIONS

Project hired members: Dr. **Ninon ROBIN** was hired at the RBINS on a 3-years post-doctoral project to lead the research featured in WP1, WP4, and WP5. Dr. **Kevin REY** was hired at the VUB on a 3-years post-doctoral project to lead the research featured in WP2 and WP3. Dr. **Séverine PAPIER** was hired at the UMONS on a 1-year research assistant position to support the research performed in WP2. **Nathalie De Wilde** was hired at the UGhent on an 18 months research assistant position to support the research performed in WP4.

❖ WP1. IMAGE AND SCREEN FOSSIL FECES OF SUCCESSIVE GEOLOGICAL AGES TO CHARACTERIZE THEIR PRODUCERS

Collecting fossil feces. The studied fossil feces from four European and Middle-East dinosaur-associated environment were collected on site and loaned by Ninon ROBIN from French, Spanish, Belgian and Lebanese collections. All are Early Cretaceous in age, and succeeding each other in time. They correspond to:

- The **140 myo** Angeac-Charente deposit (France)
- The **127 myo** Bernissart deposit (Belgium)
- The **120 myo** Bkassine deposit (Lebanon)
- The **100 myo** Teruel deposit (Spain)

The Angeac-Charente fossil feces also were also subject to additional on-field collections over which Drs. **Ninon ROBIN** and **Kevin REY** contributed in 2021 and 2022. Finally, the fossil feces from Lebanon initiated the organization of an ambitious field campaign on the Bkassine fossil site in 2023 (over a first prospective) and 2024, awarded by the *National Geographic Society* for funding and gathering a team of 10 paleontologists, including the latter researchers and PARADI²S promotor **Pascal Godefroit**. As a results, all fossil feces were studied in a context where associated animal assemblages could be thoroughly assessed – and sometimes discovered for the first time thanks to recent fieldwork.

Partial and total analyses of fossil feces. The contents of hundreds of fossil feces (**Fig. 1**) were studied in the PARADI²S program, among which 130 specimens that were analyzed *in toto* using in X-ray micro-tomography (**Fig. 2, 8**), allowing for the screening of their internal inclusions. Sub-parts of a total of 90 fossil feces were sampled for sections (**Fig. 3, 8; **studied in light microscopy, µX-ray fluorescence and LIBS spectroscopies). Another set of 40 fossil feces were sub-sampled for dissolutions and extraction of their inclusions (**Fig. 8**).

Characterizing the producers of the fossil feces. The copro-assemblages of three out of four localities were analysed and described in dedicated studies (one published, one in prep, one in a master thesis (**Fig. 8**).

That of the 140 myo fossil feces (**Angeac**, **France**) concludes on a minimum of four different original producers (1). While the Angeac-Charente biota comprised animals of a range of different ecologies, including water primary inhabitants (turtles, crocodylomorphs, actinopterygian fishes) and a variety of dinosaurs, the coprolites, together with other ichnological evidence, such as bite marks, seem to largely illustrate the **activity of the crocodylomorphs** in this ancient swamp. That of the 127 myo fossil feces (**Bernissart, Belgium**) seems to encompass two types of different producers likely being **crocodylomorphs and other undetermined archosaur** (dinosaur relatives, (2); (**Fig. 4**)). The assemblage of the 125 myo fossil feces (**Bkassine, Lebanon**) went assigned to **a variety of fishes, some of them showing intriguing structural features** diagnostic of an unknown non-spiral intestine, which we keep trying to retrieve among modern fish analogous, and to **one unidentified tetrapod**. However, the fourth (**Teruel, Spain**) assemblage of coprolites being less diverse, it was not characterized in terms of producers. Other west-Tethyan assemblages dated (Late Triassic to mid-Cretaceous in age) were, since 2024, either formally described by other research teams, or carried out to our knowledge so we could also analyzed them partially (e.g. Poland south basins, Kem-Kem formations in Morocco; Persac fluviatile deposit in France) and enrich our comparative coprolite-producer database.

Produced articles:

1. Rozada L., Allain R., Qvarnström M., **Rey K.,** Vullo R., Goedert J., Augier D. **Robin** N. 2024 A rich coprolite assemblage from Angeac-Charente (France): a glimpse into trophic interactions within an Early Cretaceous freshwater swamp. *Cretaceous Research.*
2. **Robin N., Rey K., Kaskes P., Baele J.-M., Claeys P., Godefroit P. In prep.** The coprolite assemblage of the iguanodons of Bernissart (Early Cretaceous of Belgium): 150 years later. *Fossil Record.*

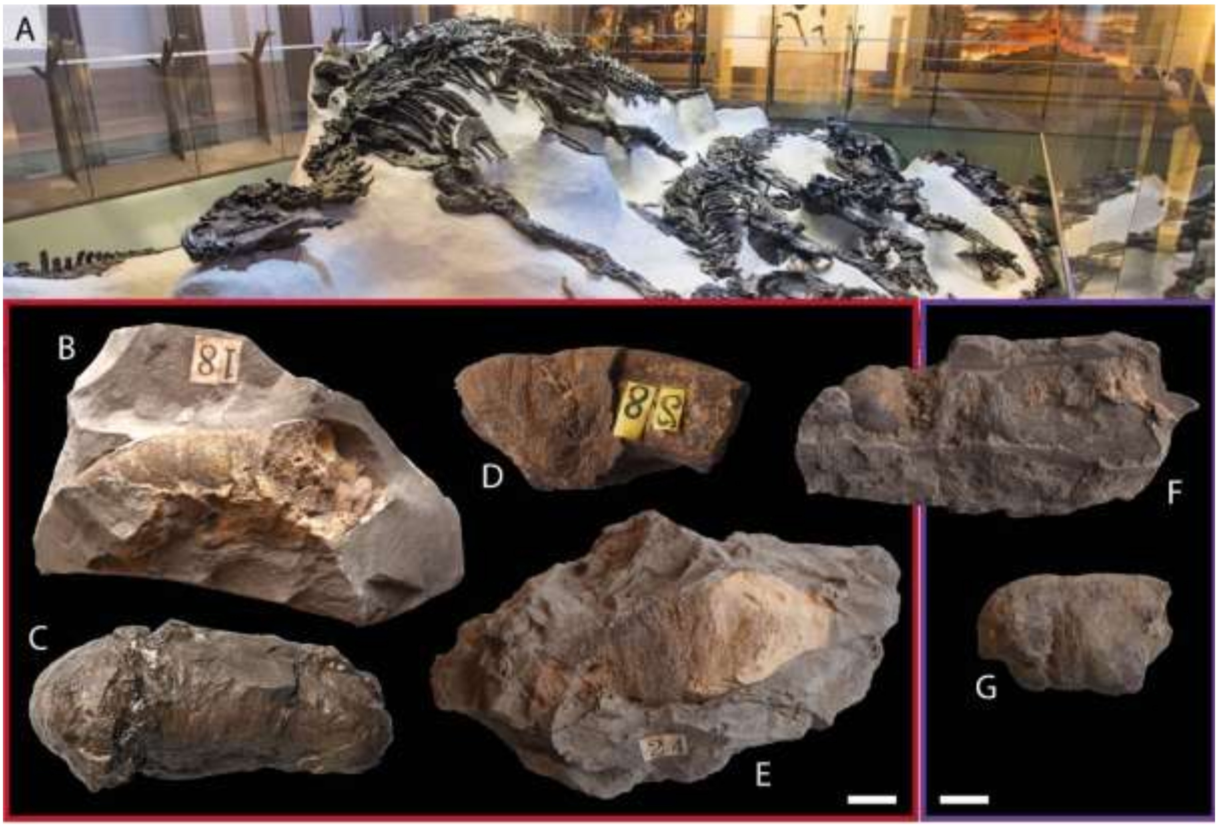

Figure 1. The example of the fossil faeces preserved with the Bernissart iguanodons. *A. The Iguanodons skeletons on display at RBINS. E-G. Example of the large to very large fossil Early Cretaceous fossil faeces associated with the skeletons. The latter were studied in tomography, thin sectioning, and soft dissolutions. Scale bars = 2 cm.*

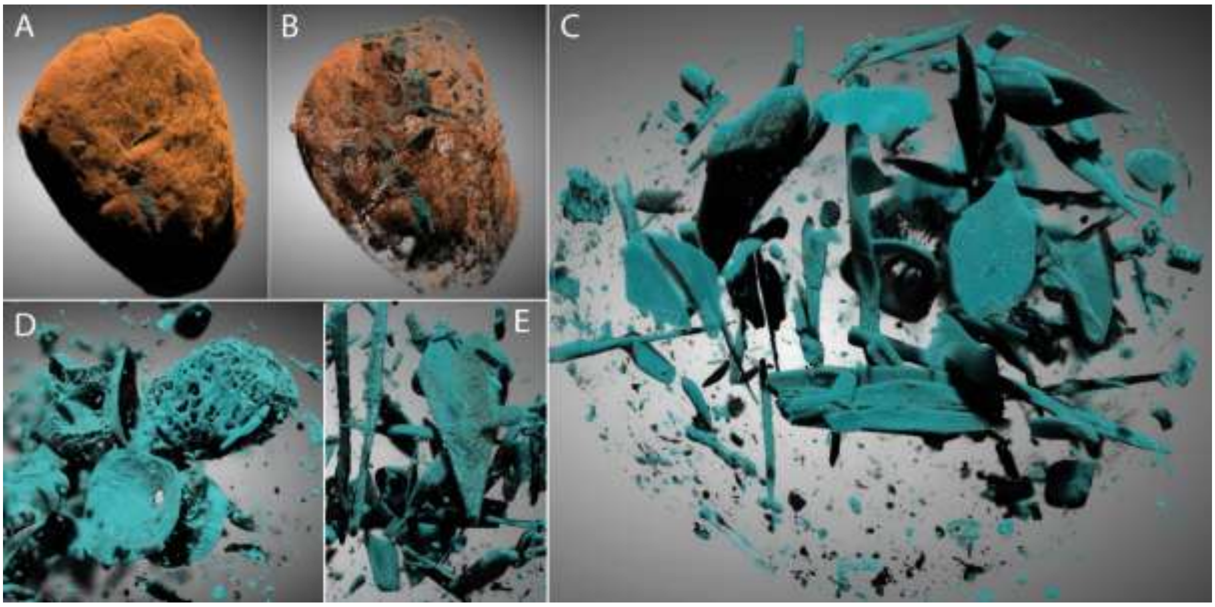

Figure 2. 3D renderings of a 127 Myo Bernissart fossil faeces studied in tomography*. A. External view of the coprolite, B. Internal view revealing diet inclusions. C. Bone remains of a preyed* Scheenstia *fish (Ginglymodi), D. vertebrae, E, scales and elongated rays.*

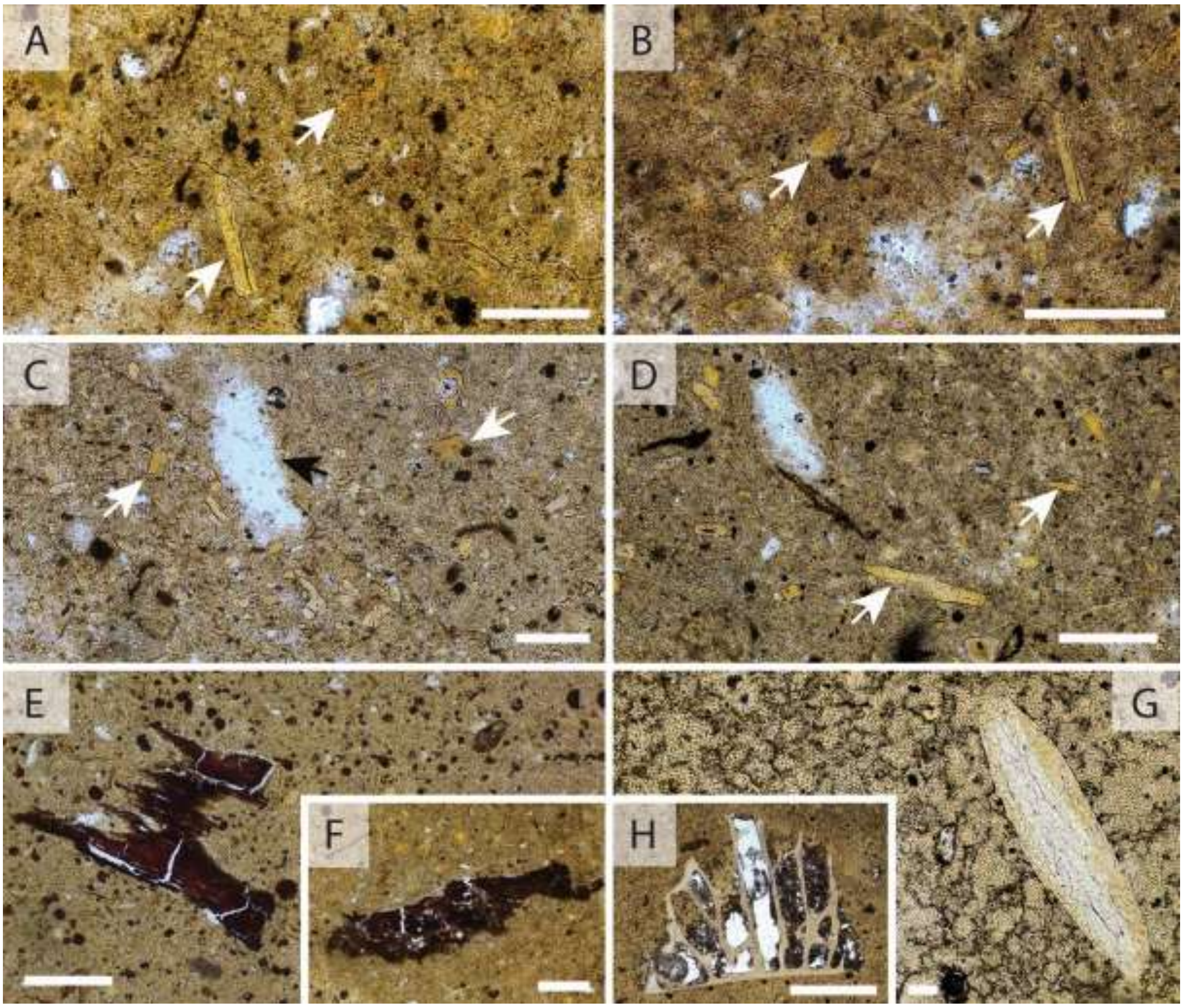

Figure 3. Thin sections in the matrix of a 127 Myo Bernissart fossil faeces*. A-D. Collagene fibers(arrows); E-F wood remains, G-H, bone remains. Scale bars = 20 µm.*

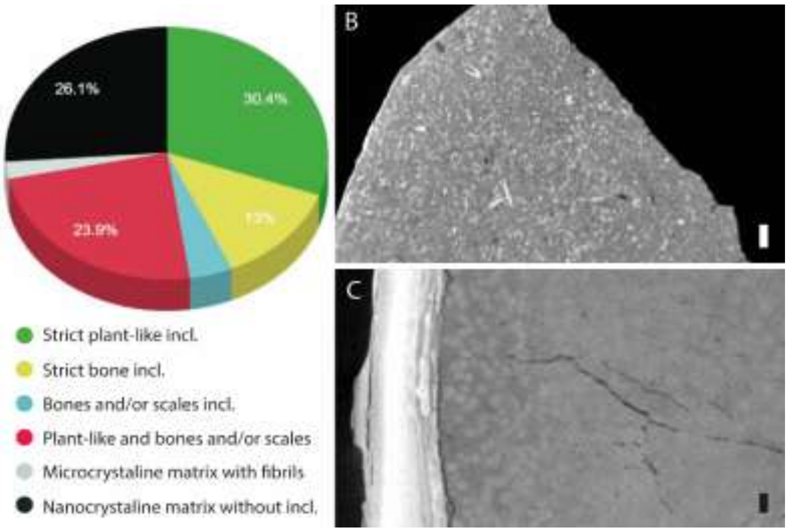

***Figure 4. Distributions of the inclusion profiles observed in the coprolites of Bernissart, Belgium** and matrices devoid of inclusions. Scale bars = 1 mm*

❖ WP2. ASSESS THE ELEMENTAL CHEMISTRY OF FOSSIL FECES FOR POSSIBLE USAGES

Coprolites are relatively poorly known fossil objects. Like most other fossil soft-tissues remains, they are often preserved in **carbonate-fluorapatite**. However, they tend to be much more common and abundant than other soft-tissues. Therefore, we questioned how much these objects can be used as **proxies for (a) producer diets, and (b) burial conditions** based on their chemical signatures. We therefore performed µXRF spectrometry, on a variety of fossil feces, in order to determine the relative and absolute elemental compositions of these objects and the distribution of the different elements within these fossils. The following sampling was assessed (**Fig. 8**):

- 50 coprolites from the Angeac-Charente deposit (France)
- 100 coprolites from the Bernissart deposit (Belgium)
- 15 coprolites from the Bkassine deposit (Lebanon)
- 30 coprolites from the Teruel deposit (Spain)

A first study has focused on the assemblage of fossil feces from Angeac-Charente that had been described in (**3**) ; it addresses the following :

- (1) do variations in the elements composing coprolites was varying depending on their different morphologies (shape and size)?
- (2) how were distributed the elements that have progressively replaced the organic matter composing ancient feces over their fossilization (i.e. over the decay, the burial and the hardening in sediments)?
- (3) how do inclusions fossilize compare to the fecal matrix?

Our study evidenced that elements of clear detrital origin (Si, Al, K) are enriched in the margin of the fossil feces, pointing toward an early surficial contamination by siliciclastic particles (**Fig. 5**). Ce, Y and Sr result from inwards migration of mobile elements initially adsorbed onto the surrounding clays and their substitution for calcium within the CFAp lattice (**Fig. 5**). Coprolite central regions are more affected by elements like Fe, S, As and Zn, reflecting the reducing conditions under which pyrite minerals precipitated. The rapid hardening of the coprolite margin by permineralization has increased reducing conditions in the center of the fecal matter, favoring precipitation of sulphide minerals. Coprolite composition therefore results from the reduction/oxidation conditions during early diagenesis, rather than from recent oxidative front, overprinting possibly existing chemical variations between different morphologies/producers. Only the smallest morphotypes are geochemically distinct from the others, with higher concentrations of Ca, compatible with a piscivorous diet. This study reveals that the chemical composition of the **coprolites reflects the burial environment rather than the specific composition of the digested food**.

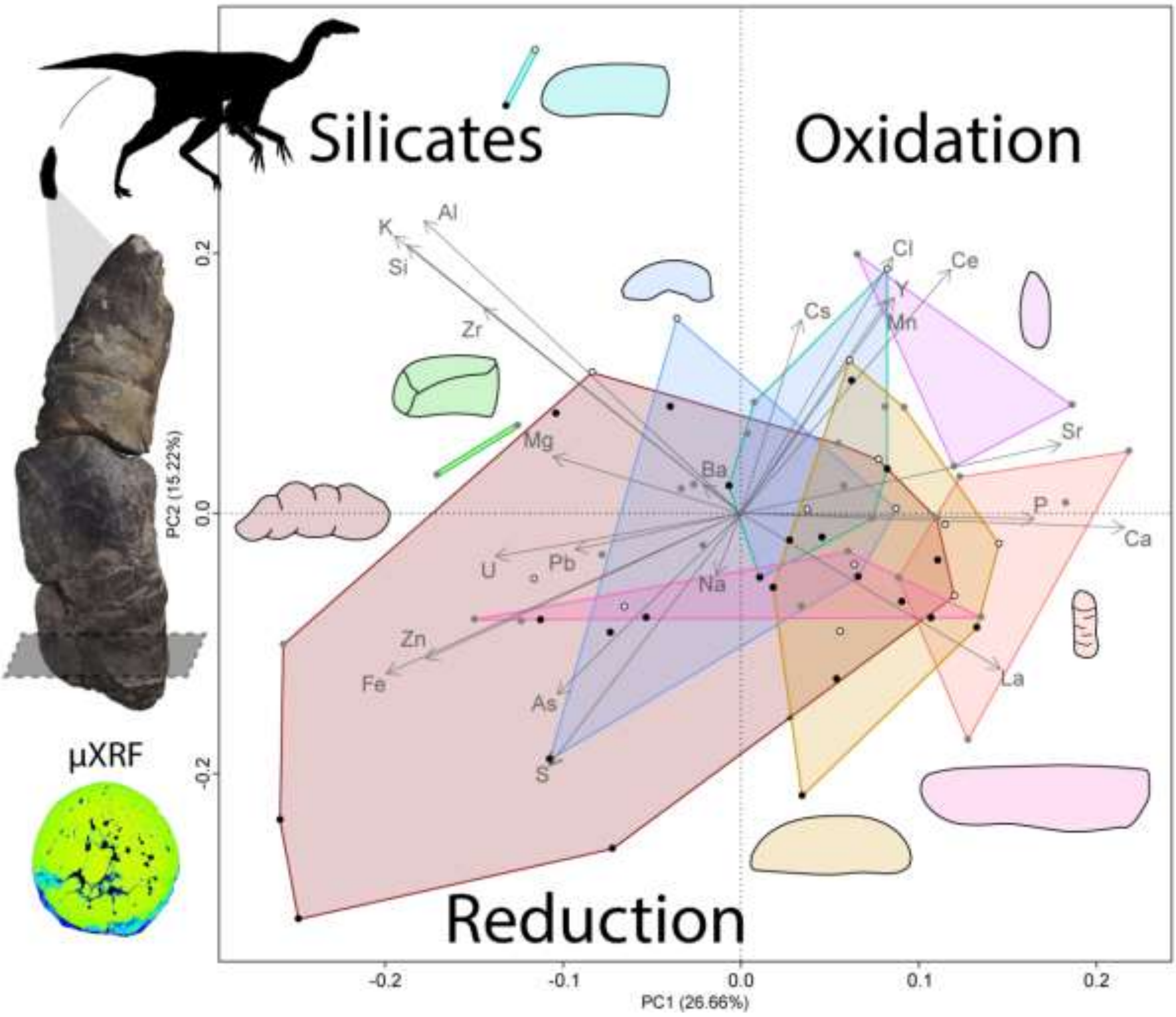

Figure 5. Elemental chemistry of fossil faeces evidenced by X-ray fluorescence spectrometry. *The figure presents an example of the 50 studied fossil afeces from the 140 Myo deposit of Angeac-Charente, France, a spectroscopic map of a section of the fossil, and a principal component analysis of the elements composing different morphologies of fossil faeces (in different colours).*

Produced articles:

2. **Robin N., Rey K., Kaskes P., Baele J.-M., Claeys P., Godefroit P. In prep.** The coprolite assemblage of the iguanodons of Bernissart (Early Cretaceous of Belgium): 150 years later. *FOSSIL RECORD.*

3. **Rey K., Robin N., Kaskes P.,** Rozada L., Allain R., **Baele J.-M., Stamataki E., Snoeck C., Claeys P. In press.** Coprolite fossilization processes revealed by micro-X-ray fluorescence (µXRF): analysis of a unique Early Cretaceous bonebed. *GEOLOGICAL JOURNAL.*

Another study (**2**) applied µXRF to the assemblage of fossil faeces from the Bernissart deposit, this time comparing the evolution of elements concentrations from coprolites inside to adjacent and remote sediment. This assemblage reflects four size categories of approximately comparable shapes (small, average large, very large). Bernissart coprolites are enriched in Sr, Y, Ce and U. Adjacent sediments are inversely richer in Si, Al, K, Ti, Zr, typical element for silicates sediments, such as clay. Sediments in Bernissart show specific concentrations, with high S and Fe concentrations and low Mg concentrations, highlighting reducing conditions. These reducing conditions are however – and unlike observed in the Angeac-Charente older deposit – absent from the fossil faeces. Out of the various faeces sizes categories, the smaller coprolites record higher Y, Ce and U concentration than larger ones. The latter elements are often added to the apatite through substitution during recrystallization, indicating that larger proportions of the smaller coprolites were affected by recrystallization. We suggest that this is explained by the fact that smaller size is more prone to let diagenesis impact fossil faeces at their centers, compared to larger objects. This pattern is also observed at the Angeac-Charente locality where the smaller faeces morphotypes (smc, and potentially ms) show higher concentrations in Y and Ce.

❖ WP3. ASSESS THE ISOTOPIC SIGNAL OF FOSSIL FECES FOR POSSIBLE PALEOENVIRONMENTAL RECONSTRUCTIONS

Another question that we addressed was how much fossil faeces can be used as **proxies for (c) paleoenvironnemental inferences** (aridity, vegetal cover, temperatures) based on their stable isotope signals. To test this, calcium-phosphates and carbonates were sampled from vertebrate skeletal remains (200 specimens) and fossil faeces (90 specimens) in each of the four studied terrestrial Early Cretaceous deposits to compare values in coprolites with those in skeletons and teeth (**Fig. 8**).

We projected both the δ^{18}Ophosphates and δ^{18}Ocarbonates values along a plot (**Fig. 6**). Each studied locality plots next to each other with very few overlapping, while coprolites values completely overlap with those from the bones and teeth (**Fig. 6**). Bernissart data are lower on the global samples line, suggesting an alteration of the phosphate or carbonate values in this deposit. For the other localities, the collected data seem to reflect the original values registered in either the bones or the faeces - when produced.

This good overlap is very interesting as it shows that **coprolites could indeed be used for paleoclimatic interpretations** like other, so far used, vertebrate remains. However, the various analyzed faeces morphotypes have overlapping values, making impossible to recognize a faeces type (and then one original producer) based on their isotopic signals. As for the case of the Angeac-Charente locality, the large number of coprolites sampled allowed the analyzes of both the center and the margin of several of them. Results show that δ^{18}Ophosphate values from the center are significantly higher than those from the margin (**Fig. 6**). This difference, consistent with µXRF data, suggest that the margin of a fossil dropping would bear a slightly altered isotopic signal; implying that the sampling for isotopic analysis should preferably be done at coprolite center. These results will be the object of a dedicated publication (**Rey et al., in prep**).

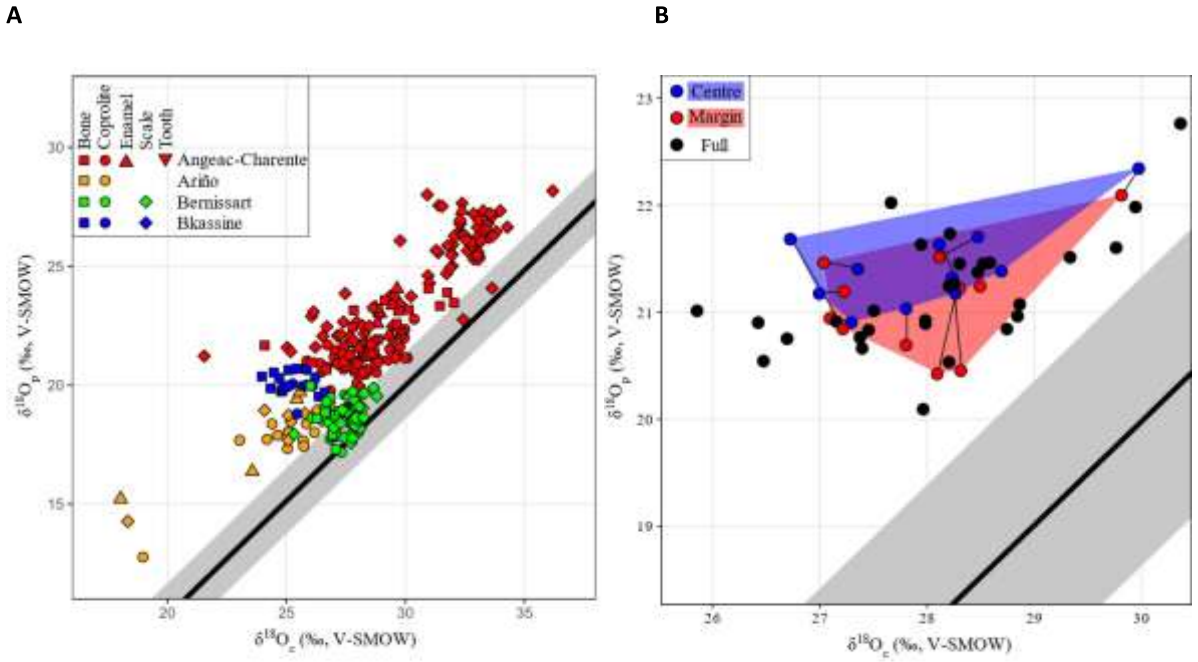

Figure 6. Oxygen isotopic values for various fossil faeces. *A. δ^{18}Ophosphate values plotted against their corresponding carbonate δ^{18}Ocarbonate values, from coprolites and vertebrate remains from lower Cretaceous localities.* **B.** *δ^{18}Ophosphate values plotted against their corresponding carbonate δ^{18}Ocarbonate values, from the Angeac Charente locality. Empirical isotopic equilibrium line with a slope close to unity (bold line).*

❖ WP4. IDENTIFY FOSSIL ENDOPARASITIC INCLUSIONS AND DEFINE THE HOST RANGES OF ANCIENT HELMINTHS

The project had aimed at collecting hundreds of endoparasitic inclusions from screened coprolites. A total of only 30 parasite-related inclusions were collected from sub-sampled portions of dissolved coprolitic matrices from Angeac-Charente and Bernissart (**Fig. 7**). A limit which appeared to researchers was the resemblance in the fossil faeces between the structures that had been so far described as fossil eggs (see Barrios-de Pedro, S., Osuna, A. & Buscalioni, Á.D. Helminth eggs from early cretaceous faeces. Sci Rep 10, 18747 (2020). https://doi.org/10.1038/s41598-020-75757-4) and a diversity of pollens (e.g. of gnetal conifers) and comparable non-pollen palynomorphs (NPPs). Consequently, members of the project decided to re-orient their question on the following : **how to recognize with confidence a 120 Myo nematod or flatworm egg from other structures present in faeces ?**

To this aim they designed a project of experimental taphonomy aiming to compare at SEM the resulting morphology (electronic microscopy) and chemistry (FTIR) of decayed parasites and pollens. The research project would be conducted on the longer term in collaboration with taphonomists from Géosciences Rennes, France – in collaboration with UGhent and RBINS. This research collaboration ran over the last part of the PARADI2S BRAIN 2.0 project, from 2023 to 2025. A diversity of nematod and flukes eggs (*Fasciola, Paramphistomum, Nematodirus, Ascaridida, Heterakis*) and adults of cestod and nematod worms (*Ascaris, Ascarididia, Raillietina, Capillaria*) were selected, searched, extracted and prepared by Nathalie De Wilde (UGhent) in order to prepare this – now new – project to come (**Fig. 7**).

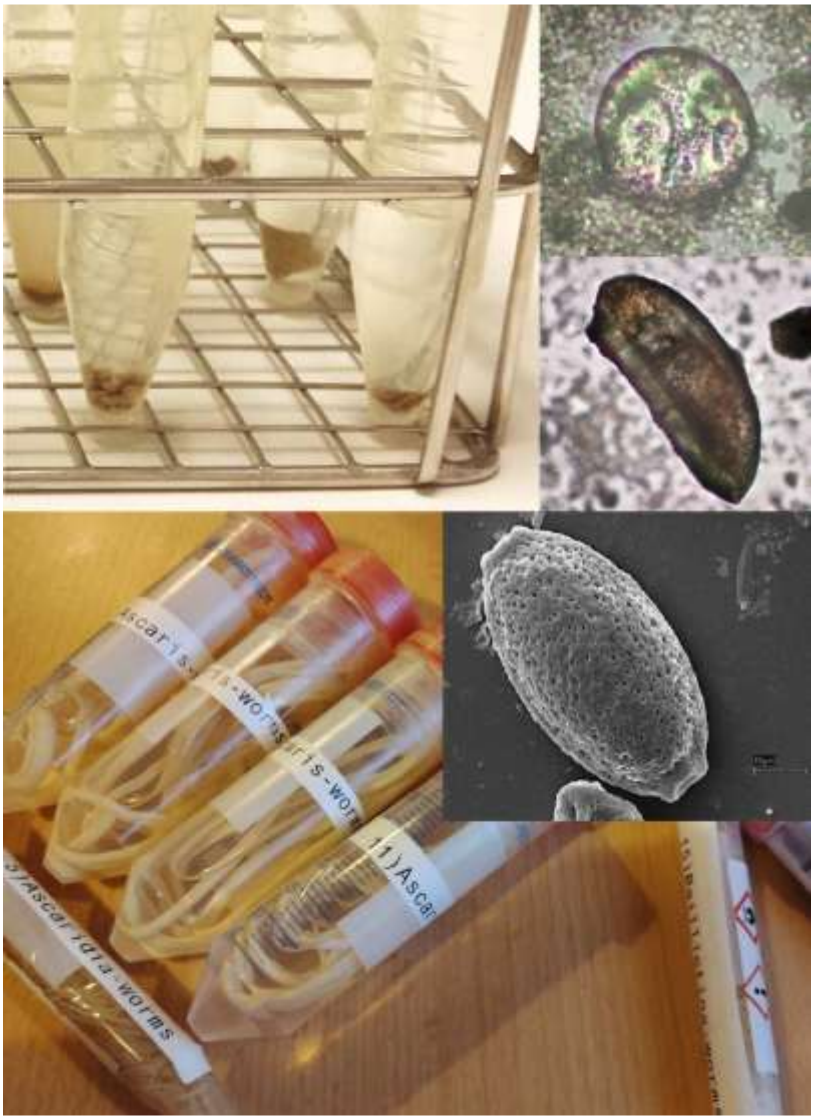

Figure 7. Identifying fossil parasitic inclusions in the Mesozoic. *Top panel: Experiences of acid light dissolution of Early Cretaceous fossil faeces and examples of fossil egg-like structures recovered from it (Bernissart, Belgium; 127 Ma). Bottom panel: modern material of comparison from UGhent (adult worm in tubes and egg under electronic microscopy).*

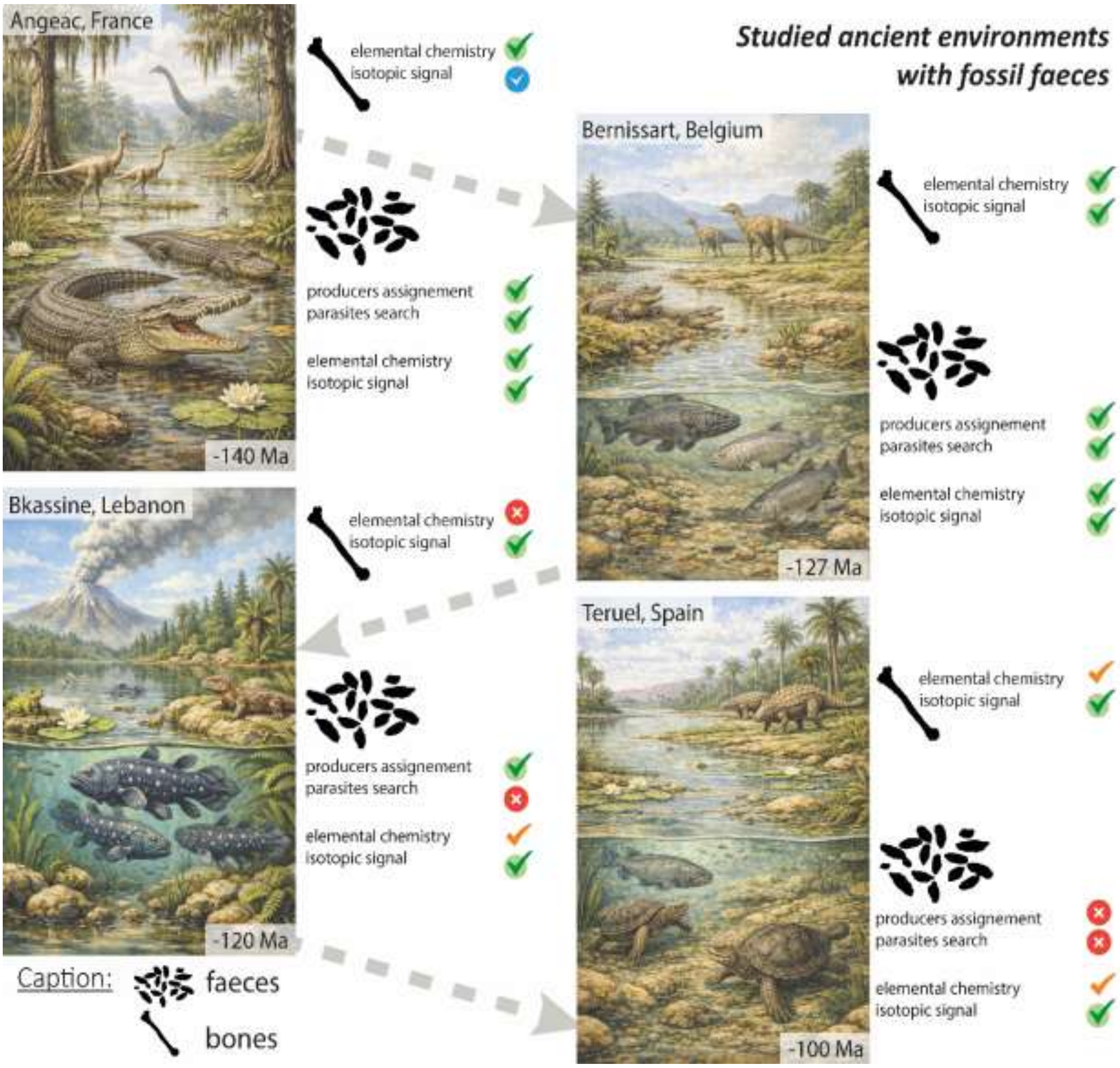

***Figure 8. Sum-up of the different PARADi²S tasks realized from WP1 to WP4**. Green check = published or to be published by PARADi²S project members, orange check = results to possibly exploit in the future, red cross = cancelled, irrelevant; blue check = made available by other projects than PARADI²S. Arrows represent the succession of the four paleoenvironments over time. Indicative vignettes result from generative AI.*

❖ WP5. ANALYSES OF EARLY CRETACEOUS ECTOPARASITIC ARTHROPODS

The visit of the Jehol Biota collections that offer important collections of dinosaurs and mammals with skin, feathers and furs could not be performed in the dedicated time (postdoc Ninon ROBIN having to quit the project earlier than expected). As a consequence, fossil parasitism was searched on other material :

- Ambers including feathers (Lebanon, Spain and French Cretaceous ambers)
- Amber record of parasites of non-vertebrates

Therefore, no large scale screenings of homogeneously preserved vertebrate tissues could be performed, only punctual descriptions of preservation, involving specific pairs of animal partnerships were carried out. The Cretaceous Lebanese amber provided interesting cases of among the oldest examples of animal interactions preserved in amber, that were the focus of dedicated studies. We report (not published) cases of :

(1) Several Mesophtirinae (Homoptera, Xylococcidae), a controversed group of insects thought for some time to be dinosaur parasites

(2) Erythraeidae mites on some 125 Myo sauropsids (Fig. 9).

Figure 9. Report of an erythraeid mite parasitic of 125 Myo sauropsid vertebrates*, preserved in Lebanese amber, bottom left showing an modern equivalent of the studied mite.*

(3) A dedicated published study (4) on the oldest association of animals preserved in amber.

The latter involved early astigmatid mites on some 125 Myo flying termites. The termite bears 16 phoretic tritonymphs of Schizoglyphidae (*Plesioglyphus lebanotermi* gen. et sp. n.). The mites are primarily attached to the membranes of the host's hindwings, using their attachment organs, pretarsal claws and tarsal setae. Additionally, we report new modern phoretic tritonymphs of this same family, on one of the earliest lineages of termites. Notably, phoretic schizoglyphids retain a distinct mouth and pharynx, whereas these structures are absent in the modern phoretic stages of non-schizoglyphid Astigmata. The discovery of Schizoglyphidae mites in Lebanese amber represents the oldest known continuous association between acariform mites and their hosts. This finding demonstrates the long-term evolutionary significance of phoresy in Astigmata, evidencing a relationship sustained for over 125 Ma. It indicates that these early mites lived inside termite nests as inquilines and used alate termites for dispersal. This ancient association offers key insights into the coevolution of both mites and termites, highlighting a potential for the future discoveries of similar mites. This fossil — a stem-group Astigmata — is important for the accurate calibration of acariform mite phylogenies, advancing our understanding of these mites evolutionary history (Fig. 10).

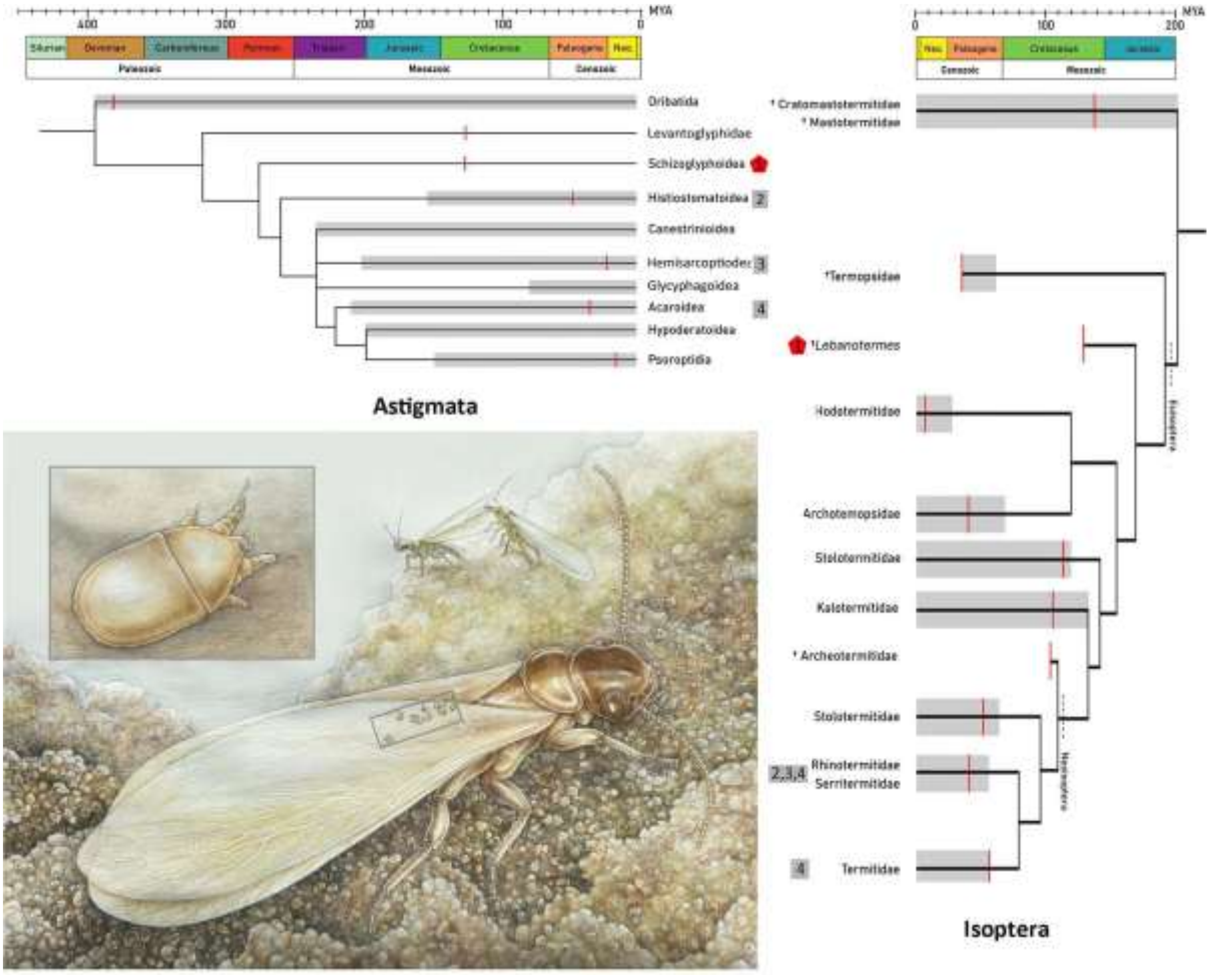

***Figure 10**. **Simplified phylogenies of Astigmatan mite and termites** correlated on a geological time scale with marked termite-mite associations and a reconstruction of the described termite-mite association. The reconstruction was made by Júlia Káčerová.*

Ectoparasitic associations with vertebrates were also studied through attempts in identifying hemoglobin presence in different types of potential blood-feeding insects. To do so, we studied three cases of insects from Lebanese and Myanmar ambers, which showed evidences either of possible blood cells preserved on mouthparts, or obvious liquid engorgments (Fig. 11). We tested for the hemglobin species of the iron contained in these blood-like structures (Fig. 11). In this purpose, Fe was mapped in X-ray fluorescence through the amber and then studied with X-ray Absorption Near Edge Structure spectroscopy at the SOLEIL Synchrotron (Orsay, France) to be ultimately compared with other types of minerally- and organically-retrieved Fe species (Fig. 11). All collected results are not indicating a clear hematoglobin-Fe speciation within studied fossil, opening questions on if the reducing conditions in amber may allow for the maintenance of hematoglobin-Fe specific bounds over time (Fig. 11).

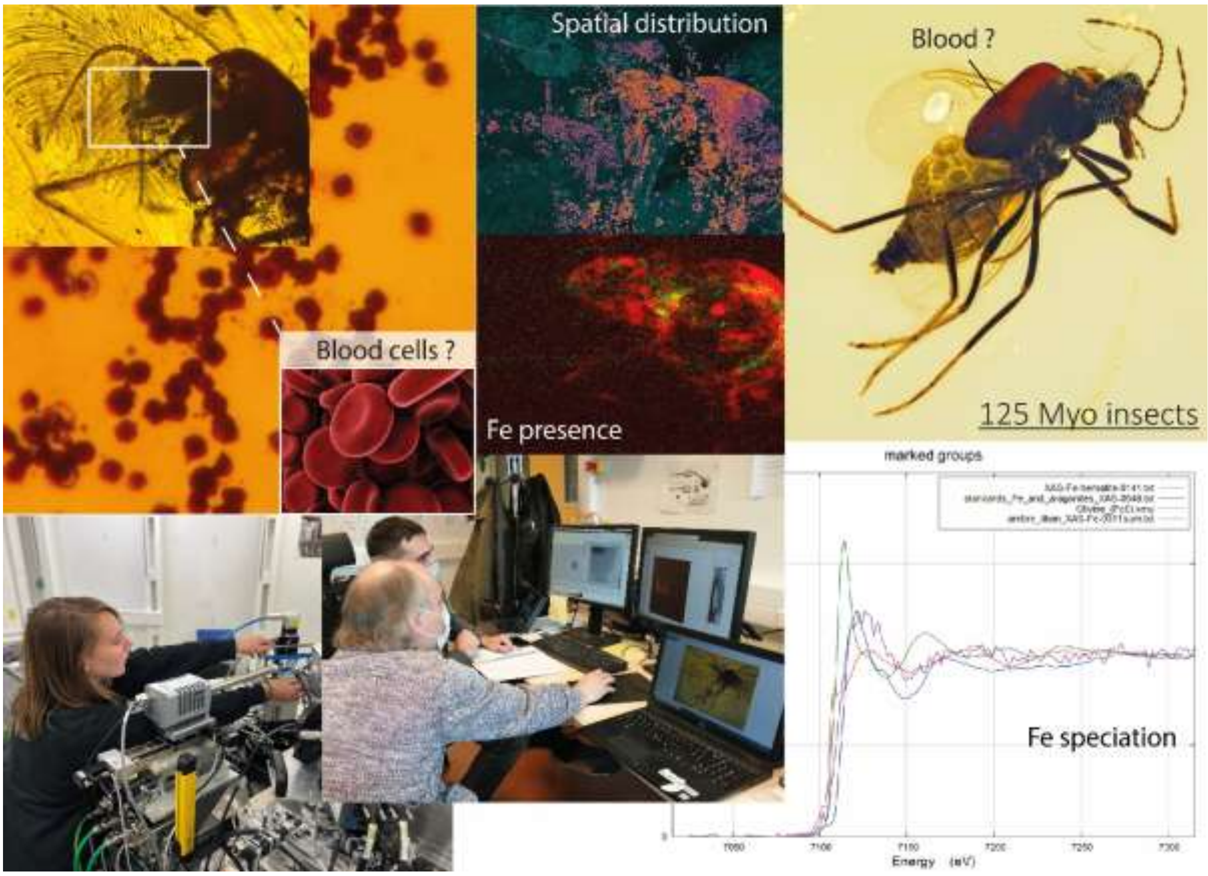

Figure 11. Studied Early Cretaceous parasitic insects preserved in amber and search for hemoglobin hints. *Left upper corner showing a fly-related insect (125 Myo Lebanese amber) with possible preserved blood cells on perforating mouthparts. Central top: tomography and Fe map on at structure locations. Right upper corner showing a fly-related insect (100 Myo Myanmar amber) with leaked brown engorgement surrounding the thorax. Left down corner: PARADI²S researcher working on the detection of 100 Myo hemoblogin remains at PUMA beamline, SOLEIL synchrotron, Orsay, France; Down right corner showing XANES spectra of compared Fe species.*

Produced articles:

4. Sendi H., Klimov P., **Bonino E.**, Azar D., **Robin N.** 2025. The oldest continuous association between astigmatid mites and termites preserved in Cretaceous amber reveals the evolutionary significance of phoresy. *BMC ECOLOGY AND EVOLUTION.*

❖ WP6. PALEOBIODIVERSITY OF A NEWLY EXCAVATED EARLY CRETACEOUS TERRESTRIAL DEPOSIT FROM THE EAST-GONDWANA

An uplanned consequence of the research carried out in WP1, was the exploration of a particularly interesting 125 Myo terrestrial deposit, co-eval of the Bernissart Belgian deposit, in the African portion of the ancient supercontinent Godwana. Located in central Lebanon, this deposit and associated localities, extremely rich in coprolites of vertebrates, was propsected in 2021 in the PARADI²S framework, in order to constrain its diversity in vertebrate animals. The prospections indicated that more diversity (Fig. 12) was to be discovered in these sedimentary layers, calling for way more ambitious excavations which ended up being supported by a Level II grant of the National Geographic Society (Fig. 12). Members of PARADI²S happened to be particularly involved and ended up conducting some “paleobiodiversity” side-research (5 and 6) acknowledging this initial BELSPO Brain 2.0 PARADI²S project.

Figure 12. Paleobiodiversity of the Bkassine (Jezzine, central Lebanon) 125 Myo fossil deposit. *The deposit involves a diversity of newly discovered fossil vertebrates potentially at the origin of the studied Early Cretaceous faeces (represented by silhouettos, coelacanths, sharks, frogs, turtles, lizards, teleostean and holostean fishes). Left bottom, the excavation team on site in the Jezzine scenic context.*

Produced articles:

5. Schädel M., Azar D., El Hajj L., Maksoud S, **Robin N. 2025.** A 130 million-year-old freshwater isopod shining new light on the origin of groundwater Cymothoida. *Royal Society Open Science.*

6. Azar D., Maksoud S., **Robin N., Godefroit P.,** Cavin L., **Olive S., Rey K., Vallée-Gillette N., De Brito L.,** Heneine G., Nel A. 2024. The first and oldest record of the bug family Issidae (Hemiptera) from the Lower Cretaceous of Lebanon. *Zootaxa* 5562(1), 38-44

5. DISSEMINATION AND VALORISATION

- **Dissemination of results to scientific audience.** Results went regularly shared to the scientific paleobio-/geology communities through the participation to professional meetings and scientific peer-reviewed publications (see below).

<u>**Abstracts of communications in international symposia**</u> <u>(in bold are the involved Belgian collaborators)</u>

1. J. Anquetin*, N. Bouchema, **N. Vallée-Gillette,** R. Roch, **S. Olive, N. Robin, P. Godefroit**, L. Cavin, S. Maksoud, D. Azar **2025**. A new Pan-Chelidae (Testudines, Pleurodira) from the Early Cretaceous of the northern margin of Gondwana (Lebanon). *85th Annual meeting of the Society of Vertebrate Paleontology*, Birmingham, UK **(P)**.

2. **Rey, K.*,** Goedert, J., **Robin, N.,** Rozada, L., Allain, R., **Godefroit, P., Claeys, P. 2025.** Isotopic analysis of coprolites (Early Cretaceous of France): a new paleoenvironmental and paleoecological tracker? *17th congress of the European Society for Isotope Research*, Lyon, France. **(O)**

3. **Vallée-Gillette N.*,** Azar D., **De Brito L.,** Cavin L., **Godefroit P.,** Heneine G., Maksoud S., **Olive S., Rey K, Robin N. 2025**. From Field to Lab : preparation of vertebrate fossils from a new Barremian Lagerstätte in South Lebanon. *30th Symposium on Preparation and Conservation,* Leiden, Pays-bas. **(O)**

4. Azar D.*, Maksoud S., **Robin N., De Brito L., Godefroit P., Olive S.,** Cavin L., **Vallée-Gillette N., Rey K,** Heneine G. **2025**. The lower Barremian Bkassine (South Lebanon) dysodile Konzentrat and Konservat-Lagerstätte: insights on the fossil continental assemblage. *Fossil insects – bridging past & present*, Kraków, Pologne. **(O)**

5. Azar D.*, Maksoud S., **Robin N., De Brito L., Godefroit P., Olive S.,** Cavin L., **Vallée-Gillette N., Rey K,** Heneine G. **2024**. The lower Barremian Bkassine (Lebanon) dysodile Konzentrat and Konservat-Lagerstätte: insights on the fossil continental assemblage. *37th International Geological Congress,* Busan, Corée du Sud. **(O)**

6. Cavin L.*, **Godefroit P., Robin N., Olive S., Vallée-Gillette N., Rey K., De Brito L.,** Yuan Z., Heneine G., Maksoud S., Azar D. **2024.** A coelacanth pond in the Early Cretaceous of Lebanon. *22nd Swiss Geoscience Meeting*, Basel, Switzerland. **(O)**

7. Azar D.*, Maksoud S., **Robin** N., De Brito L., Godefroit P., Olive S., Cavin L., **Vallée-Gillette N., Rey K,** Heneine G. **2024**. The Bkassine dysodile Konzentrat-Lagerstätte from the lower Barremian of Lebanon: fossil continental assemblage and reconstruction of their depositional environment. *7th International Conference of Fossil Insects, Arthropods and Amber*, Xi'an, Chine. **(O)**

8. **Kaskes P.*, Rey K., Robin N., Baele J.-M., Godefroit P., Claeys P. 2024**. Revisiting the Bernissart Iguanodon Bonebed in Belgium: new taphonomic and stratigraphic insights based on trace element geochemistry and carbon isotopes. *200 Years of Dinosaurs: New Perspectives on an Ancient World - Conference*, Londres, UK. **(O)**

9. **Rey K.*, Robin N., Kaskes P.,** Rozada L., Allain R., L. **Baele J.-M., Stamataki E., Claeys P**. **2023**. Chemical characterization of an Early Cretaceous coprolite assemblage (Angeac-Charente, France): feces fossilization informed using XRF element mapping. *Annual Goldschmidt Conference,* Lyon, France. **(O)**

10. **Rey K.*, Robin N., Kaskes P.** Rozada L. **Baele J.-M., Claeys P. 2022**. Chemical characterization of an Early Cretaceous coprolite assemblage (Angeac-Charente, France): feces fossilization informed using XRF element mapping. *6th International Palaeontological Congress*, Khon Kaen, Thailande. **(O)**

- **Dissemination by museums, universities, and deposits local authorities.** Valorization were performed by the different local authorities and fossil curation teams; a particular echo was given in media on the Lebanese-National Geographic excavations through museum webpages and Lebanese media.

6. PUBLICATIONS

Papers in IF journals (in bold are the involved Belgian collaborators)

1. **Robin N.**, Nattier R., **Locatelli C.**, Seyfullah L. Perrichot V., Sendi H, Nel. A. **In press.** More from the Eocene Belgian amber: the oldest ladybird of the genus *Scymnus* Kugelann (Coleoptera: Coccinellidae). *PALEOENTOMOLOGY*. (IF: 1.4)

2. **Rey K., Robin N., Kaskes P.,** Rozada L., Allain R., **Baele J.-M., Stamataki E., Snoeck C., Claeys P. In press.** Coprolite fossilization processes revealed by micro-X-ray fluorescence (µXRF) analysis of a unique Early Cretaceous bonebed. *GEOLOGICAL JOURNAL*. (IF: 2.0)

3. Jiadong H., Wenhao W., Lei M., Bertozzo F., Dhouailly D., **Robin N.**, Pittman M., Kaye T., Manucci F., Xuezhie H., Xuri W., **Godefroit P. 2026.** An Early Cretaceous iguanodontian dinosaur with histologically-preserved spike-like cutaneous appendages. *Nature Ecology and Evolution* (IF: 14.1)

4. Boderau M., Schubnel T., **Mottequin B., Robin N.**, Garrouste R., Mazurier A. , Ngô-Muller Valérie, Nel A. **In press.** Revisiting the wing venation in Hemiptera: mutual insights from the earliest representatives and 3D vein reconstruction. *PAPERS IN PALEONTOLOGY*. (IF: 2.2)

5. Santos A.A., McLoughlin S., **Mottequin B., Robin N.**, Nel. A. **2025.** Old collections, new taxa: late Carboniferous roachoids ('Dictyoptera') among plants with insect interactions from the Tangshan Formation, China, stored in European museums. *PALAEOENTOMOLOGY*. (IF: 2.3)

6. Schädel M., Azar D., El Hajj L., Maksoud S, **Robin N. 2025.** A 130 million-year-old freshwater isopod shining new light on the origin of groundwater Cymothoida. *ROYAL SOCIETY OPEN SCIENCE*. (IF: 3.0)

7. Sendi H., Klimov P., **Bonino E.,** Azar D., **Robin N**. **2025.** The oldest continuous association between astigmatid mites and termites preserved in Cretaceous amber reveals the evolutionary significance of phoresy. *BMC ECOLOGY AND EVOLUTION*. (IF: 3.3)

8. Azar D., Maksoud S., **Robin N., Godefroit P.,** Cavin L**., Olive S., Rey K., Vallée-Gillette N., De Brito L.,** Heneine G., Nel A. **2024.** The first and oldest record of the bug family Issidae (Hemiptera) from the Lower Cretaceous of Lebanon. *ZOOTAXA* 5562(1), 38-44 (IF: 0.9)

9. Nel A., Sendi H., **Robin N.**, **Mottequin B. 2024.** Late is not too late: formal descriptions of Carboniferous insects from Western Europe studied by Daniel Laurentiaux (Palaeodictyoptera, Paoliida). *PALAEOENTOMOLOGY*. (IF: 2.3)

10. Rozada L., Allain R., Qvarnström M., **Rey K.,** Vullo R., Goedert J., Augier D. **Robin N. 2024** A rich coprolite assemblage from Angeac-Charente (France): a glimpse into trophic interactions within an Early Cretaceous freshwater swamp. *CRETACEOUS RESEARCH*. (IF: 2.2)

11. **Mottequin B., N. Robin. 2023.** Rediscovery of the Mathieu collection of Carboniferous (Pennsylvanian)–Permian (Cisuralian) arthropods from the Kaiping Coalfield (northeastern China). *GEOLOGICA BELGICA* 26/1-2. (IF: 1.8)

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