

Insect Imaging at the ANKA Synchrotron Radiation Facility

Bildgebung von Insekten an der Synchrotronstrahlungsquelle ANKA

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Summary: Internal structures of biological samples are often difficult to visualize by traditional morphological methods like light and electron microscopy. In insects, a robust cuticle often impedes examination. Three-dimensional visualization of anatomical details based on light microscopy photographs is particularly challenging, because the necessary creation of a series of “perfect” slices often proves to be impossible in the case of hard-shelled specimens. Synchrotron-based X-ray imaging provides a pool of techniques well-suited for morphological studies. As it allows examining millimeter-sized non-translucent objects, it is particularly valuable for the multidimensional visualization of insects and became increasingly popular among entomologists. A synchrotron is a cyclic particle accelerator. In high vacuum electrons are accelerated up to nearly light speed, injected into a storage ring and deviated by bending magnets. When the electron beam changes its direction due to magnetic influence, electromagnetic radiation is transmitted tangentially, which is used in attached experimental stations (“beamlines”). Synchrotron radiation has a broad spectrum ranging from microwaves to hard X-rays, the latter being used for most synchrotron imaging techniques. The high intensity of these X-rays facilitates high special and temporal resolutions. An important method is synchrotron X-ray microtomography (SR- μ CT). Here, a series of 2D X-ray projections of a rotating sample is used to calculate a 3D volume. This technique is already widely employed for the three-dimensional imaging of insects.

Visualization is usually done by volume renderings or by the creation of surface models based on labeled structures inside the data set, allowing moving body parts with respect to each other in order to simulate joints. Automated motion estimation techniques may help to analyse time-resolved data by providing quantitative dynamical information. During the last years, several insect-biased experiments have been performed at the TOPO-TOMO beamline of the ANKA synchrotron radiation facility (Angströmquelle Karlsruhe) operated by the Karlsruhe Institute of Technology. One focus of the experiments was the investigation of weevil morphology. By using SR- μ CT it was shown that the hip joints of *Trigonopterus oblongus* resemble pairs of screw (trochanter) and nut (coxa). Tomography of fossil weevils from Baltic amber revealed morphological details that could not be identified visually. The development of specialized detector systems significantly reduced exposure times, thus facilitating scans of alcohol-preserved and living insects. The head of the stick insect *Peruphasma schultei* was successfully tomographed while being in ethanol. High-speed radiography (2D movies) helped analyzing the mating of tsetse flies and the feeding process of cockroaches. Further, a new method called X-ray 4D cine-tomography was developed, combining ultra-fast X-ray tomography and sophisticated image analysis. Using this technique it was possible to track the real movement of a living weevil’s screw joint in space and time. Imaging techniques available at ANKA will be further improved. For this purpose, a powerful new beamline (IMAGE) is currently in construction.

Keywords: X-rays, synchrotron, radiography, microtomography, image analysis

Zusammenfassung: Innere Strukturen biologischer Proben sind oft nur mühsam mithilfe traditioneller Methoden der Morphologie, wie Licht- und Elektronenmikroskopie, darzustellen. Bei Insekten werden morphologische Untersuchungen zudem häufig durch eine robuste Cuticula erschwert.

Insbesondere die dreidimensionale Visualisierung anatomischer Details basierend auf lichtmikroskopischen Aufnahmen stellt häufig ein Problem dar, da die dazu notwendige Herstellung „perfekter“ Schnittserien im Falle von hartschaligen Objekten oft nicht zu bewerkstelligen ist. Synchrotronbasierte Röntgen-Bildgebung bietet eine Reihe von Techniken für morphologische Untersuchungen. Da sie sich besonders für undurchsichtige, millimetergroße Objekte eignet, ist sie für die mehrdimensionale Visualisierung von Insekten besonders wertvoll und wird inzwischen regelmäßig für entomologische Studien verwendet. Ein Synchrotron ist ein Teilchenbeschleuniger, der Elektronen im Hochvakuum auf nahezu Lichtgeschwindigkeit beschleunigt. Die Elektronen werden in einen Speicherring injiziert und durch Magneten in der Spur gehalten. Wenn der Elektronenstrahl abgelenkt wird, wird tangential elektromagnetische Strahlung emittiert, die in angeschlossenen Messstationen („Beamlines“) genutzt wird. Das Spektrum der Synchrotronstrahlung reicht von Mikrowellen bis hin zu harter Röntgenstrahlung, die üblicherweise bei bildgebenden Verfahren verwendet wird. Die Intensität synchrotronbasierter Röntgenstrahlung ist sehr groß und erlaubt daher eine hohe räumliche und zeitliche Auflösung. Eine wichtige Methode ist die Synchrotron-Mikro-Computertomographie (SR- μ CT), bei der aus einer Reihe von 2D-Röntgenbildern einer rotierenden Probe ein 3D-Volumen berechnet wird. Sie wird bereits seit einigen Jahren sehr erfolgreich zur dreidimensionalen Darstellung von Insekten eingesetzt. Die Visualisierung der Daten erfolgt üblicherweise durch sogenannte Volumenrenderings oder mit Hilfe von Oberflächenmodellen basierend auf markierten Strukturen innerhalb eines Datensatzes. Auf diese Weise können Körperteile virtuell gegeneinander bewegt werden, beispielsweise um die Bewegung von Gelenken zu simulieren. Bei der Analyse zeitaufgelöster Daten kann zusätzlich eine computergestützte Bildanalyse quantitative Informationen zur Dynamik liefern. An der TOPO-TOMO-Beamline der Synchrotronstrahlungsquelle ANKA (Angrstromquelle Karlsruhe) des Karlsruher Instituts für Technologie wurden während der letzten Jahre einige entomologische Experimente durchgeführt. Ein Schwerpunkt lag dabei auf der Untersuchung der Morphologie von Rüsselkäfern. Im Rahmen der Studien konnte mit Hilfe von SR- μ CT nachgewiesen werden, dass die Hüftgelenke von *Trigonopterus oblongus* wie Paare aus Schraube (Trochanter) und Mutter (Coxa) funktionieren. Die Tomographie fossiler Rüsselkäfer in Baltischem Bernstein erlaubte die Darstellung morphologischer Details, die bei einer visuellen Inspektion nicht erkennbar waren. Durch die Entwicklung eines spezialisierten Detektorsystems konnte die Belichtungszeit stark reduziert und Scans von Alkoholpräparaten oder auch lebender Insekten ermöglicht werden. So wurde der in Ethanol konservierte Kopf der Stabschrecke *Peruphasma schultei* erfolgreich tomographiert. Mittels Hochgeschwindigkeits-Radiographie (2D-Filme) konnte die Paarung von Tsetse-Fliegen und die Nahrungsaufnahme von Schaben analysiert werden. Außerdem wurde die neue Methode der Röntgen-4D-Cine-Tomographie entwickelt, die Hochgeschwindigkeitstomographie mit einer hochentwickelten Bildanalyse kombiniert. Mittels dieser Technik war es möglich, die räumliche Bewegung des Schraubengelenks eines lebenden Rüsselkäfers in Echtzeit aufzunehmen. Die bildgebenden Methoden an der ANKA werden in Zukunft weiter optimiert werden. Zu diesem Zweck befindet sich eine neue, leistungsfähige Beamline (IMAGE) im Aufbau.

Schlüsselwörter: Röntgenstrahlung, Synchrotron, Radiographie, Mikrotomographie, Bildanalyse

1. Introduction

Due to the penetration ability of the radiation, X-ray imaging is highly appropriate to visualize internal morphological characters of opaque millimeter-sized specimens. It is non-destructive and therefore applicable to a broad spectrum of samples (MIZUTANI & SUZUKI 2011), especially to those that are not cuttable or of considerable value.

In recent years, synchrotron-based X-ray imaging has been established as an important tool in entomology to approach a wide range of scientific questions. It was applied to study the physiology of tracheal respiration (e. g. WESTNEAT et al. 2003; SOCHA et al. 2007), anatomic characteristics (e. g. DE ALMEIDA et al. 2012; KIM et al. 2012), the functional morphology of exoskeletal joints (VAN DE KAMP et al. 2011) and to

examine inclusions of fossil insects in amber (e. g. TAFFOREAU et al. 2006; POHL et al. 2010; SORIANO et al. 2010; PERREAU & TAFFOREAU 2011).

A synchrotron (Fig. 1) is a cyclic particle accelerator. In high vacuum, electrons are accelerated up to nearly light speed and injected into a large storage ring. In order to keep the electrons on their course they are deviated by bending magnets. Every time the electron beam changes its direction due to magnetic influence, synchrotron radiation is emitted tangentially, which enters experimental stations through so-called beamlines. Synchrotron radiation consists of a broad spectrum of electromagnetic radiation from the microwave range to hard X-rays. The intensity of synchrotron-based X-rays is several orders of magnitudes higher than from laboratory X-ray tubes. The small size, its small divergence and the micrometre fo-

cusings allow investigating even very small samples with high spatial resolution below 1 μm (BAUMBACH et al. 2009; RACK et al. 2010). Modern synchrotron radiation facilities provide brilliant and partially coherent radiation for fast 2D and 3D volumetric imaging methods such as synchrotron-based X-ray computed microtomography (SR- μCT ; Fig. 2). The latter technique allows three-dimensional imaging of millimeter-sized organisms (FRIEDRICH & BEUTEL 2008; WEIDE & BETZ 2008) and is already considered a “methodological milestone in microanatomical research” (BETZ et al. 2007, p. 68).

The ANKA synchrotron radiation facility (Angströmquelle Karlsruhe; Fig. 3A, B) is operated by the Karlsruhe Institute of Technology (KIT) and started user operation in 2003. Fourteen beamlines are operational, three more are in commissioning or design phase, including a new imaging beamline

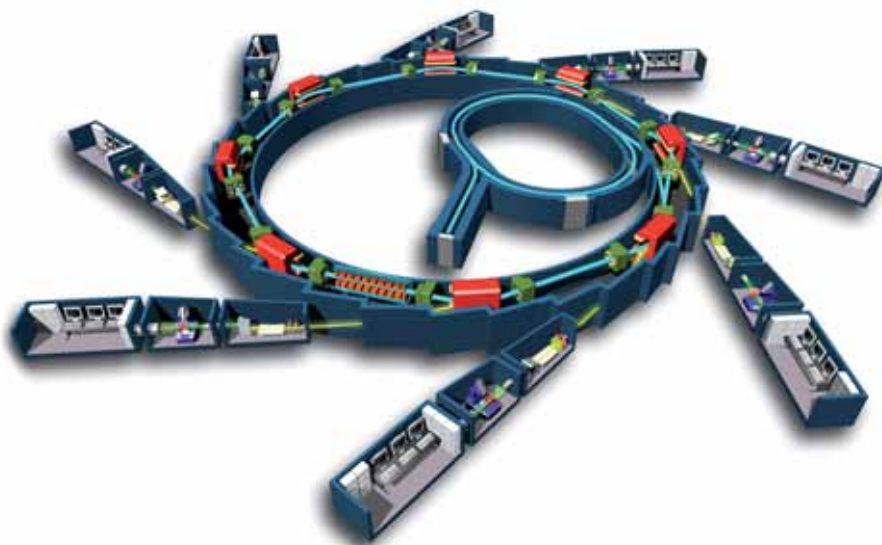


Fig. 1: Scheme of a synchrotron light source. Electrons (blue) are accelerated, injected into the storage ring and deviated by bending magnets (red). Electromagnetic radiation (yellow) is emitted tangentially and used in attached experimental stations.

Abb. 1: Schema einer Synchrotronstrahlungsquelle. Elektronen (blau) werden beschleunigt, in den Speicherring injiziert und durch Magnete (rot) abgelenkt. Elektromagnetische Strahlung (gelb) wird tangential emittiert und in angeschlossenen Experimentierhütten eingesetzt. Copyright © EPSIM 3D/JF Santarelli, Synchrotron Soleil.

(IMAGE). The synchrotron is composed of a 500 MeV injector and a 2.5 GeV storage ring with 110 m circumference (Fig. 3B). During user operation, a maximum current of 180 mA is circulating after injection. As a large-scale research facility, ANKA provides beamtime for fundamental and application-oriented research to international users free of charge (HEINRICH et al. 2011). Methods provided are versatile and range from infrared/THz and X-ray spectroscopy over

various X-ray diffraction and imaging techniques to X-ray lithography.

Synchrotron X-ray imaging becomes increasingly popular in life sciences, and – alongside experiments in molecular and developmental biology – several insect-related experiments were performed lately at ANKA. Because of their small size, many insects fit well in the field of view of X-ray detector systems and therefore constitute adequate samples for synchro-

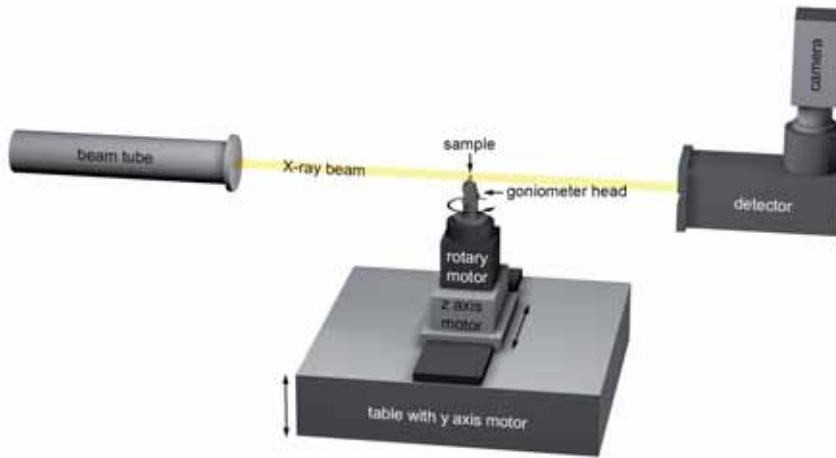
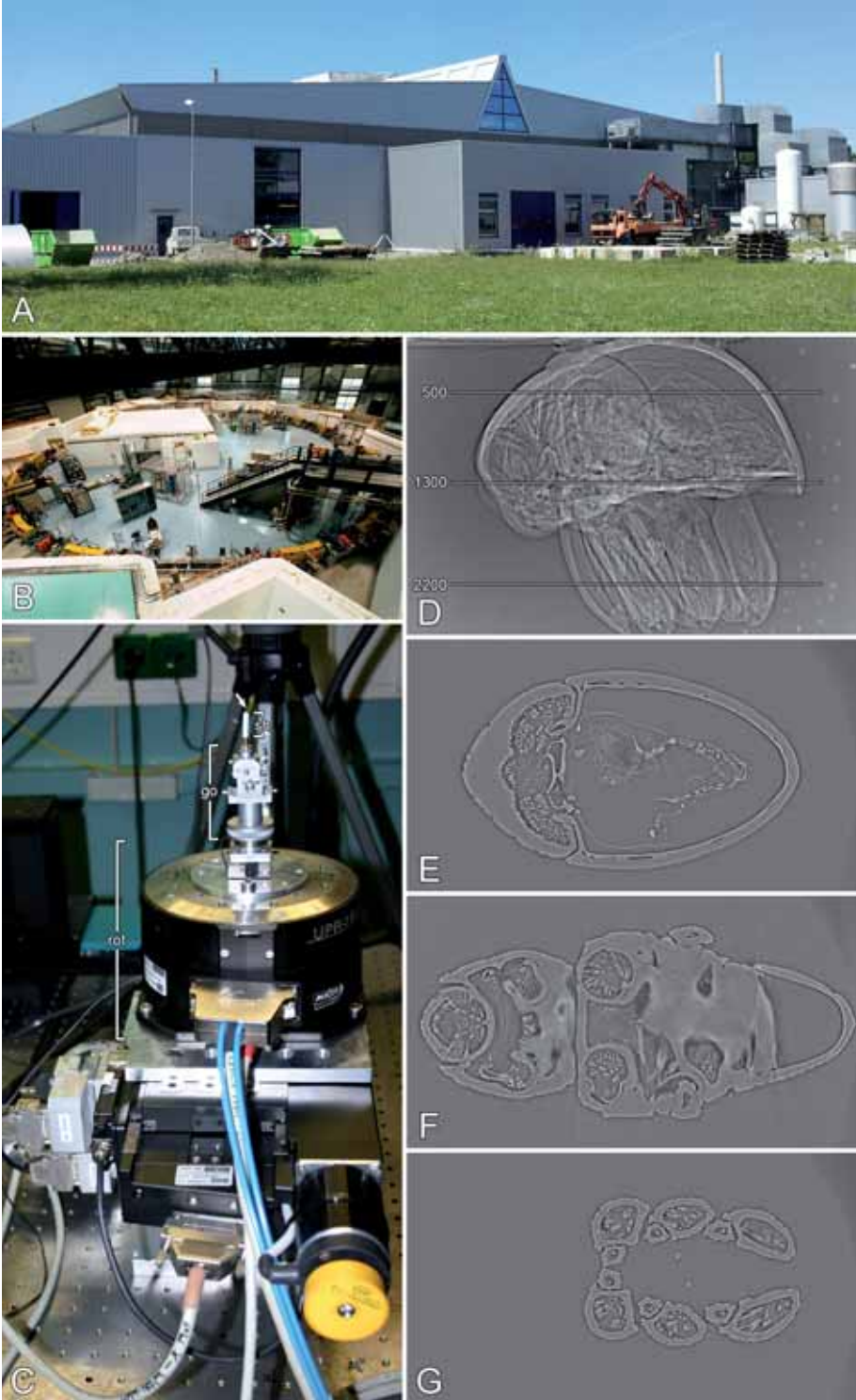


Fig. 2: Scheme of a setup for X-ray microtomography. X-rays travelling from left to right pass through the sample mounted on a goniometer head on rotary stage. While the sample is rotating, a detector system converts X-rays to visible light that is subsequently recorded by a digital camera. **Abb. 2:** Schema eines Röntgen-Mikrotomographie-Aufbaus. Röntgenstrahlen treffen auf durchleuchten die Probe, die über einen Goniometerkopf mit einem Rotationsmotor verbunden ist. Während sich die Probe dreht, wandelt ein Detektorsystem die Röntgenstrahlen in sichtbares Licht um, das von einer Digitalkamera aufgezeichnet wird.

Fig. 3: X-ray microtomography at ANKA. **A** The ANKA hall at Karlsruhe Institute of Technology. **B** View of the interior of the ANKA hall with the accelerator complex. The storage ring with 110 m circumference is located close to the inner side of the concrete radiation protection wall. **C** Sample (arrow) at the rotary stage inside the experimental station of the TOPO-TOMO beamline. **D** Radiograph of a *Trigonopterus* weevil; lines indicating cutting slices through the resulting volume. **E-G** Virtual slices through the volume corresponding to the lines shown in D (E: 500; F: 1300; G: 2200). go = goniometer head; rot = rotary stage; sh = sample holder with attached specimen (arrow). **Abb. 3:** Röntgen-Mikrotomographie an der ANKA. **A** Die ANKA-Halle am Karlsruher Institut für Technologie. **B** Blick auf das Innere der Halle mit Beschleunigerkomplex. Der Speicherring mit 110 m Umfang befindet sich an der Innenseite einer Strahlenschutzmauer aus Beton. **C** Probe (Pfeil) auf der Rotationsachse in der Experimentierhütte der TOPO-TOMO-Beamline. **D** Radiogramm eines *Trigonopterus*-Rüsselkäfers; Linien deuten Schnitte durch das spätere Volumen an. **E-G** Den Linien aus D entsprechende virtuelle Schnitte durch das Volumen (E: 500; F: 1300; G: 2200). go = Goniometerkopf; rot = Rotationsmotor; sh = Probenhalter mit Probe (Pfeil).



tron X-ray imaging. Herein, we will provide an overview about recent entomological experiments performed at ANKA's TOPO-TOMO beamline, including microtomography of amber inclusions, digital reconstruction of beetle hip joints and *in vivo* imaging of living insects.

2. Principles of the method

2.1. Synchrotron-based X-ray imaging

For X-ray imaging, radiation transmitted through a sample is recorded by a detector system. It consists of a crystal scintillator, which converts X-rays into visible light, relay lenses and a digital camera (Fig. 2). For a tomographic scan, a number of radiographic projections (Fig. 3D) of an angular range of 180° are acquired. These projections are used to reconstruct a tomographic volume of the sample (Fig. 3E-G). Due to partial coherence of the incoming X-rays, phase contrast is accessible in addition to conventional absorption contrast. This allows imaging of weakly absorbing samples (see BETZ et al. 2007 and WESTNEAT et al. 2008 for reviews).

2.2. Visualization of multidimensional data

Generally, there are two ways to visualize volumetric data: First, by voxel-based volume renderings, which are convenient to quickly examine whole objects, and second, by surface models based on labelled structures inside the image stack. The latter technique is time consuming, but allows distinguishing and analysing more delicate external and internal structures (e. g. FRIEDEMANN et al. 2011; WEIDE et al. 2012). The creation of polygon surface models based on 3D image stacks proved to be extremely valuable to examine aspects of the functional morphology of small biological specimens. Complex models composed

of several individual components can be adjusted to show only selected parts of interest, e. g. distinct muscle groups or parts of the skeleton. Another great advantage is the possibility to move the distinct parts with respect to each other, e. g. to analyse and visualise the three-dimensional movement of joints – externally and internally (VAN DE KAMP et al. 2011). This provides a more detailed analysis than examining the original specimen with a microscope. Further, surface models may be embedded into PDF publications or other interactive software to provide 3D data to the scientific community (RUTHENSTEINER & HESS 2008).

2.3. Image analysis

A significant advantage of X-ray based techniques is that obtained 2D or 3D digital images could be further evaluated using numerous data analysis software (e. g. SCHINDELIN et al. 2012). After morphological features in projection or tomographic data are labeled manually or using automated segmentation techniques, a wide range of quantitative information is available. This facilitates accurate measurements of anatomical features and comparative studies between different specimens.

By recording time-lapse sequences of X-ray images, physiological and morphological changes inside living insects could be investigated. A number of automated motion estimation techniques facilitate capturing of dynamical information. Popular choices are so-called optical flow methods (BROX et al. 2004; BAKER et al. 2011) which find displacements between corresponding pixels in subsequent images. The computed motion fields thus provide quantitative dynamical information about the specimen and may be further exploited to extract more sophisticated measures. Moreover, individual anatomical features of insects can be tracked to analyze their kinematics or visualize complex coordination of movements.

3. Application examples

3.1. Digital reconstruction of a biological screw joint

We investigated the leg movement of the hyperdiverse (RIEDEL et al. 2009, 2013; RIEDEL 2010) Indo-Pacific weevil genus *Trigonopterus*. Their body-size range between 1.5 and 5 mm. Due to their extremely robust exoskeleton (VAN DE KAMP & GREVEN 2010), the anatomy of the weevils could not be examined by microscopy. *Trigonopterus oblongus* (Fig. 4A), one of the largest representatives of the genus with a body length of about 5 mm, was scanned at ANKA to examine the functionality of its hip joints. The sample was fixed in ethanol and critical-point-dried. A high-resolution setup with a 10x magnifying optical microscope and a pco.4000 14 bit CCD digital camera with 4008 x 2672 pixels led to an effective pixel size of 0.9 μm .

The hip joints consist of two components, the coxa and the trochanter. After manual labelling of the joint parts and involved muscles in the tomographic volume, polygon surface models of coxae and trochanters were created to simulate their movement by interactively moving the partners against each other. It

became evident that the hip joint (Fig. 4B) of *Trigonopterus* is highly modified and combines a rotational movement with a single-axis translation. In *Trigonopterus*, the apical opening of the coxae is wide, circular, and mesally shows a notch marking the start of well-defined inner threads which continue internally for 345°. Thus, the coxae closely resemble engineered screw-nuts. The trochanters carry external spiral threads that cover 410° and are perfectly compatible with the threads in the respective coxal openings. Proximally, the trochanter is produced into a thorn that spears the entire coxa; when the hind leg is depressed, the thorn's tip penetrates the opposite coxal wall through a small opening, thus stabilizing the joint along the rotational axis and securing it against jamming. Meanwhile, the examination of other weevil genera showed similarities with the coxa-trochanteral joint of *Trigonopterus*. The screw-and-nut system appears to be widespread among weevils and may indeed represent a basic character of the family (VAN DE KAMP et al. 2011).

3.2. Tomography of amber inclusions

An important application of X-ray microtomography in entomology is the visualization

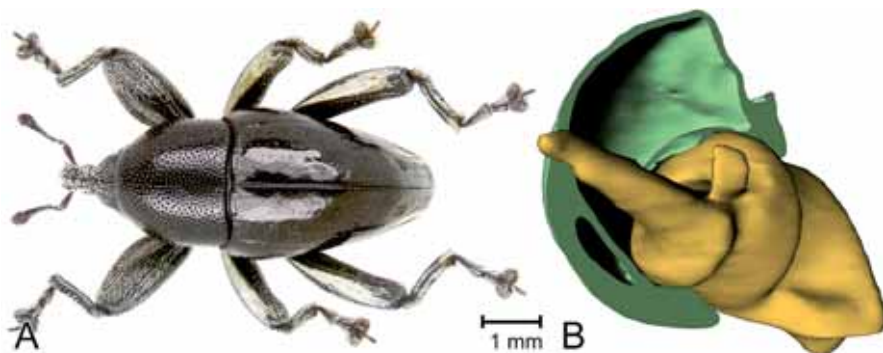


Fig. 4 Microtomographic scans of *Trigonopterus*-weevils revealed that their hip joints work as screw-and-nut systems. **A** *Trigonopterus oblongus* (photograph). **B** Surface model of the hind leg's hip joint with coxa (green) and trochanter (yellow).

Abb. 4 Mikrotomographische Scans von *Trigonopterus*-Rüsselkäfern ergaben, dass ihre Hüftgelenke wie Paare aus Schraube und Mutter funktionieren. **A** *Trigonopterus oblongus* (Foto). **B** Oberflächenmodell eines Hinterhüftgelenks mit Coxa (grün) und Trochanter (gelb).

of amber inclusions (TAFFOREAU et al. 2006; POHL et al. 2010; SORIANO et al. 2010; PERREAU & TAFFOREAU 2011). The ability to picture a three-dimensional specimen without reflections and distracting particles is of great value for taxonomic studies on extinct species. The resulting “virtual insects” can be made widely accessible to the scientific community, a clear benefit especially in the case of valuable type material that may become lost or degraded in storage over time. Hard X-rays, however, may cause darkening of the amber, thus this effect should be tested with a blank piece of the same type before scanning a valuable sample. Furthermore, epoxy resin often used to seal amber pieces may suffer heat damage during the tomographic scan.

The most convenient way of digital visualization is a greyscale-based volume rendering or surface model; the latter may be embedded into PDF documents. Depending on the sample, it is not always easy to identify the greyscales that define

the specimen as phase contrast imaging results in white-black fringes in the images (BETZ et al. 2007). Moreover, amber samples often consist of the remains of the inclusion and the negative imprint of the original insect, which are represented by different greyscales. Thus, it can be tricky to define the parameters that guarantee the best 3D visualization. Occlusions of debris may be an additional challenge. If they are separated from the specimen, they can be removed easily during post-processing, but debris or bubbles in direct contact with the specimen are more difficult and time-consuming to deal with.

We performed high resolution microtomography (same setup as described for *Trigonopterus*) of fossil weevils of the subfamily Sayrevilleinae in Baltic amber. The techniques provided important additional information about the morphological characteristics of the scanned specimens, which could not be identified by visual inspection (RIEDEL et al. 2012; Fig. 5).

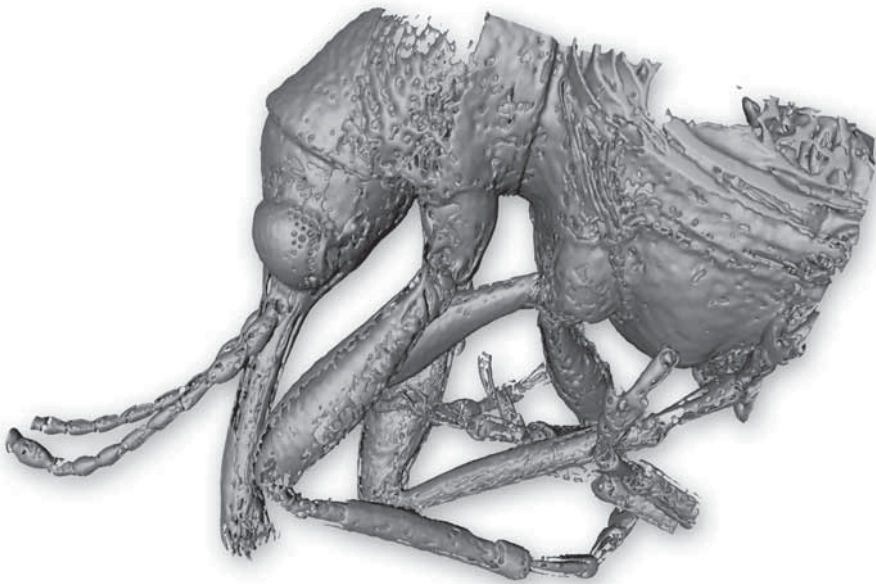


Fig. 5: 3D surface model of the fossil weevil *Baltocar hoffeinsorum* from Baltic amber.

Abb. 5: 3D-Oberflächenmodell des fossilen Rüsselkäfers *Baltocar hoffeinsorum* aus baltischem Bernstein.

3.3. Fast tomography of an alcohol-preserved stick insect

While in many cases critical-point-drying may be an adequate method to prepare an insect for X-ray microtomography, anatomical characters are usually better preserved when the specimen is fixed and stored in alcohol. When exposed to intense X-rays, however, alcohol starts boiling after a short time. Bubbles are formed in the process, which result in heavy motion artifacts compromising any tomographic scan.

At ANKA, a dedicated high-speed detector system has been designed that can withstand intense white (= polychromatic) beam. The use of white beam ensures that enough light is available to realize shortest exposure times. Frame rates of several ten thousand images per second have been achieved using the detector system in combination with a high-speed pco.dimax 12 bit CMOS digital camera with 2016 x 2016 pixels. By coupling this high-speed image acquisition with a fast, continuous rotation of the specimen, it was possible to decrease the total scan time for

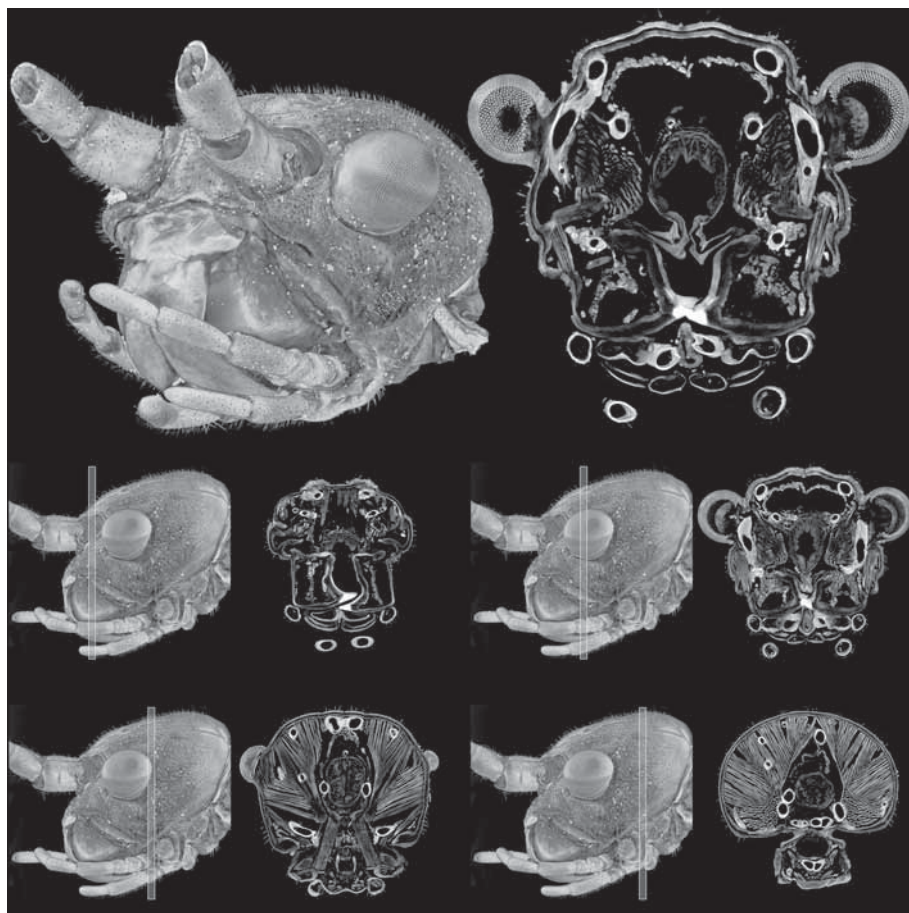


Fig. 6: Volume rendering of the head of the stick insect *Peruphasma schultei*. Thick virtual slices allow examination of anatomical details without losing the 3D impression.

Abb. 6: Volumenrendering des Kopfes der Stabschrecke *Peruphasma schultei*. Dicke virtuelle Schnitte erlauben die Untersuchung anatomischer Details, ohne dass der 3D-Eindruck verloren geht.

a complete tomogram from roughly two hours down to 400 milliseconds. Using high-speed SR- μ CT, a full tomographic scan of an alcohol-preserved sample can be performed before the medium starts boiling. It is therefore possible to examine alcohol-stored specimens while benefiting both from well-preserved anatomical details and fast throughput times.

We performed a fast tomographic scan of the head of the stick insect *Peruphasma schultei*. The sample was fixed in glutaraldehyde, stained with iodine overnight and was subsequently stored in 90% ethanol. The resulting tomographic image stack was visualized as a volume rendering and cut into thick virtual slices, allowing examination of anatomical details without losing the 3D impression (Fig. 6).

3.4. In vivo X-ray cine-radiography

While real-time examination of movements in living insects by visible light is often

challenging, because involved structures are partly concealed, X-rays overcome this problem and are therefore highly appropriate to examine internal kinematics. Cine-radiography, the recording of sequences of 2D absorption images, was successfully employed to visualize physiological processes in insects (e. g. WESTNEAT et al. 2003; SOCHA et al. 2007). Using this method, the complex kinematics of fast-moving insects was visualized at ANKA. Optical flow analysis (BROX et al. 2004) adopted for X-ray data was employed on time-lapse sequences of radiograms to capture motion fields (Fig. 7). One application was the study of the mouthparts during the feeding process of cockroaches (*Periplaneta americana*) (BETZ et al. 2008; Fig. 7A). The insects were placed on the sample manipulator and pre-aligned with visible light in order to minimize the radiation dose. They were scanned with a spatial resolution of 13 μ m and 28 μ m while acquiring up to 250 images per second using a Photron Fastcam SA-1 CMOS digital

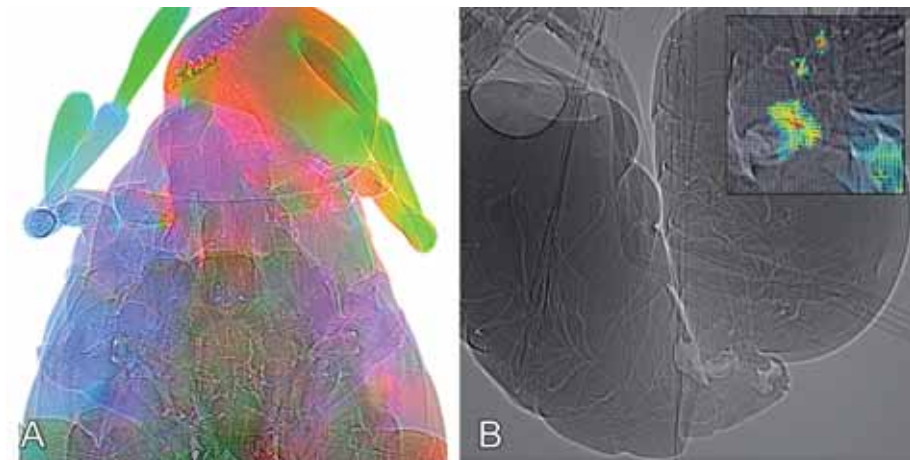


Fig. 7: Fast radiography and motion analysis. **A** Motion analysis of a feeding cockroach (*Periplaneta americana*). Colour shows particular movement direction. **B** Radiogram of the abdomens of mating Tsetse flies (*Glossina pallidipes*). Movement of the male copulatory organs is indicated by vectors colored by their magnitude.

Abb. 7: Schnelle Radiographie und Bewegungsanalyse. **A** Bewegungsanalyse einer fressenden Schabe (*Periplaneta americana*). **B** Radiogramm der Abdomina sich paarender Tsetsefliegen (*Glossina pallidipes*). Die Bewegung der männlichen Geschlechtsorgane wird durch ein Farbfeld dargestellt.

camera. It was found that movement of the mandibles is well synchronized during feeding. While the maxillary palpi act independently from each other, thus demonstrating the special role of these mouthparts, which serve as mechano- and chemoreceptors and may also help to keep the food in the median plane of other mouthparts.

Another cine-radiographic experiment was the study of Tsetse fly (*Glossina pallidipes*) genitalia during copulation (BRICEÑO et al. 2010; Fig. 7B), using a similar detector setup. The morphology of insect genitalia is often considered an important taxonomic character, but information about their

kinematics during copulation is missing. In the experiment, female flies were glued to plastic rods. Afterwards males were introduced from an adjacent chamber. The males did not take notice from the intense X-ray beam in the beginning and mounted the females undistracted. Unfortunately it was not possible to follow the complete copulation process, for the flies separated after 5-10 min. Thus, the movements of the male genitalia observed in the X-ray videos before separating of the couples are probably not directly related to sperm transfer and may rather serve to stimulate the female before ejaculation.

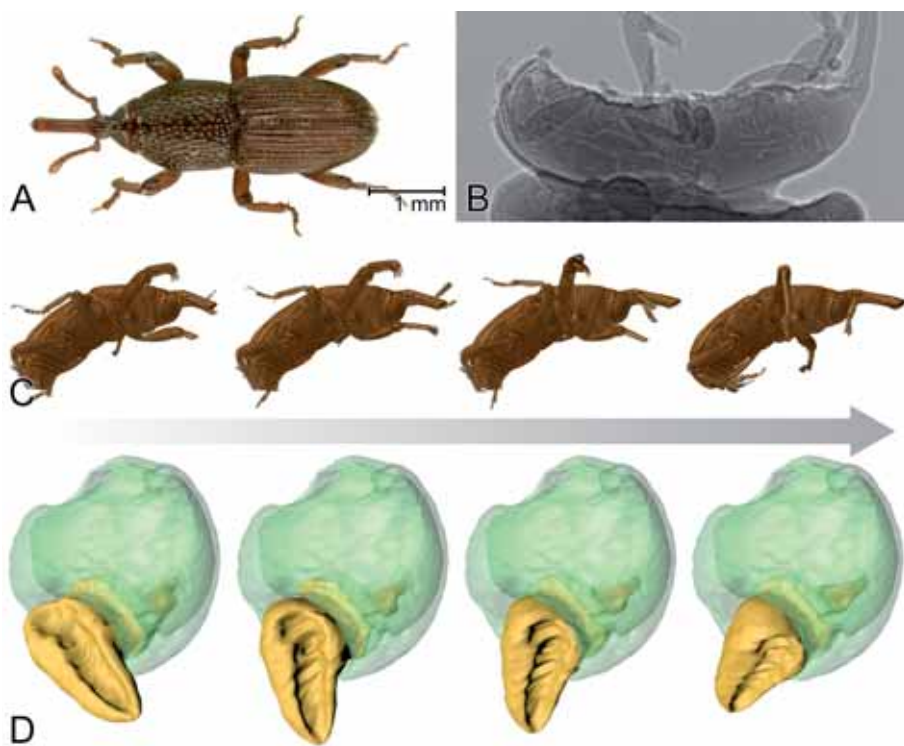


Fig. 8 *In vivo* X-ray 4D cine-tomography. **A** *Sitophilus granarius* (photograph). **B** Radiograph of living specimen. **C** Sequence of volume renderings taken from a scan of a moving weevil. **D** Animated 3D surface model showing the original movements of a hip joint in a living weevil over 665 ms (coxa semitransparent green, trochanter yellow).

Abb. 8 *In vivo* Röntgen-4D-Cine-Tomographie. **A** *Sitophilus granarius* (Foto). **B** Radiogramm eines lebenden Exemplars. **C** Sequenz von Volumenrenderings basierend auf der Aufnahme eines lebenden Rüsselkäfers. **D** Animiertes 3D-Oberflächenmodell, das die echte Bewegung eines Hüftgelenks in einem lebenden Rüsselkäfer über 665 ms zeigt (Coxa semitransparent-grün, Trochanter gelb).

3.5. In vivo X-ray 4D cine-tomography

As shown above, X-ray cine-radiography provides important real-time information about functional dynamics in living animals using sequences of 2D projection radiographs. However, this technique does not provide any information about the third spatial dimension.

To study the hitherto inaccessible 3D morphological dynamics, we developed a technique called “X-ray 4D cine-tomography” (ultra-fast X-ray tomography in combination with automated data processing and motion analysis routines), which enables the real-time study of the physiology of small animals. The setup uses an ultra-fast, high-resolution and X-ray efficient detector configuration. Data read-out is facilitated by a high-speed pco.dimax digital camera capable of detecting up to 100.000 images per second. The potential of the technique was demonstrated by the example of fast-moving screw-and-nut-type hip joints of weevils, as described on dried *Trigonopterus*-weevils before. Here, the wheat weevil *Sitophilus granarius*, was used as sample. By tracking the movement of the left hind leg hip joint, the complete 4D spatio-temporal information about its kinematics was extracted (Fig. 8). It was shown that the relation of rotary and translator movements is non-linear, facilitated by an opening between the corresponding threads that is considerably wider than in the joint described in *Trigonopterus oblongus* (DOS SANTOS ROLO et al. 2013). By realizing the fastest reported computed tomography sequences for sub-10 μm spatial resolution, X-ray 4D cine-tomography has been shown to be a promising new tool to study morphological dynamics in millimeter-sized insects.

4. Perspectives

X-rays provide a pool of versatile methods to examine insect form and function. Syn-

chrotrons are already widely used by entomologists and synchrotron-based imaging techniques are steadily improving to achieve higher image resolution, shorter acquisition times and to optimize data reconstruction and visualization.

At ANKA, a new imaging beamline (IMAGE) is currently being reconstructed, where two experimental stations will be established. Station 1 will constitute of a full-field transmission X-ray microscope for hard X-rays, allowing analysing smallest samples down to a resolution of ca. 20 nm. Station 2 will provide fast X-ray microtomography and laminography. The latter technique is a special type of tomography used for flat objects, which is very suitable to examine fossil specimen inside stone plates (HOUSSAYE et al. 2011). A sample exchange robot will facilitate high sample throughput while a reconstruction server enables on-line reconstruction of tomographic volumes. A laboratory especially designed for the preparation of biological samples was recently commissioned.

For the future we foresee more comprehensive studies on insect morphology, taxonomy and paleontology. A database will be deployed to provide scientists worldwide with three-dimensional morphological data from different insects. We plan to further establish *in vivo* X-ray 4D cine-tomography as a technique and are eager to allocate synchrotron X-ray imaging techniques to a broad base of entomologists.

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