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Cover illustration: Larval mantis shrimp *Rissoides desmaresti* (Risso, 1816), see Vansteenbrugge et al.

# EDITORIAL

Dear readers and colleagues,

It is my pleasure to inform you that, for the first time in five years, the impact factor of the Belgian Journal of Zoology has increased, to 0.531!

This has mainly been achieved by a strong selection process, filtering out unsuitable papers before refereeing. As a consequence, we have less but better papers that are published.

I also take the opportunity of the good news on the impact factor to invite you to submit your manuscripts to our journal. The Belgian Journal of Zoology welcomes reviews, opinion papers, research papers and short notes in all areas of Zoology with a focus on biodiversity and adaptations. We also consider negative results for publication. Please bear in mind that research papers should be hypothesis-driven. We can guarantee a fast, professional reviewing process and publication of your papers, with free open access. I have just handled our 938$^{th}$ submission. It would be great if we could reach 1000 submissions soon, before our 150$^{th}$ anniversary next year.

I am very much looking forward to receiving your submissions.

Isa Schön

Editor-in-Chief

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# A qualitative comparison of 3D visualization in *Xenopus laevis* using a traditional method and a non-destructive method

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ABSTRACT. Many tools are currently available to investigate and visualize soft and hard tissues in animals both in high-resolution and three dimensions. The most popular and traditional method is based on destructive histological techniques. However, these techniques have some specific limitations. In order to avoid those limitations, various non-destructive approaches have surfaced in the last decades. One of those is micro-CT-scanning. In the best conditions, resolution achieved in micro-CT currently approaches that of standard histological protocols. In addition to bone, soft tissues can also be made visible through micro-CT-scanning. However, discriminating between structures of the same tissue and among different tissue types remains a challenge. An alternative approach, which has not yet been explored to its full potential for comparative anatomy studies, is Orthogonal-Plane Fluorescence Optical Sectioning (OPFOS) microscopy or tomography, also known as (Laser) Light Sheet based Fluorescence Microscopy (LSFM). In this study, we compare OPFOS with light microscopy, applying those techniques to the model organism *Xenopus laevis*. The potential of both methods for discrimination between different types of tissues, as well as different structures of the same tissue type, is tested and illustrated. Since the histological sections provided a better resolution, adjacent structures of the same tissue type could be discerned more easily compared to our OPFOS images. However, we obtained a more naturally-shaped 3D model of the musculoskeletal system of *Xenopus laevis* with OPFOS. An overview of the advantages and disadvantages of both techniques is given and their applicability for a wider scope of biological research is discussed.

KEY WORDS: vertebrates, histological sectioning, optical sectioning, *Xenopus*, 3D visualization.

## INTRODUCTION

Three-dimensional (3D) visualization tools have been applied in biological sciences for decades (e.g. Afshar & Dykes, 1982; Johnson & Capowski, 1983; Vanden Berghe et al., 1986). Although two-dimensional images already reveal much information, a three-dimensional visualization is particularly important since development occurs in three dimensions. Reconstructions of complex anatomical structures in three dimensions are of great importance to fully grasp 3D topography of those components and to properly interpret how the structures physically interact with each other. In this way, the individual bones, cartilage, muscles, etc. can be viewed from different angles, which is necessary to correctly characterize the morphology. For this reason, there is a growing demand for 3D digital images and models. Serial histological sectioning (SHS), dissections or clearing and staining are traditional and destructive approaches to obtain morphological 3D data and information. SHS is a procedure entailing the sectioning of thin slices of a specimen or tissue in a consecutive order. The sample first has to be fixed for the preservation of the structural components, stained and embedded prior to sectioning. The sections are then imaged with an optical

microscope and can subsequently be used for microscopic examination or 3D reconstruction of anatomical structures. A drawback of SHS, however, is that it is time-consuming and relies on a destructive protocol. Moreover, when 3D reconstructions are generated based on those histological sections, manual alignment and segmenting of the sections is required prior to reconstructing.

Recently, several automated and non-destructive imaging techniques have been introduced. Those techniques are able to generate virtual serial sections in any orientation (e.g. frontal, sagittal or transversal) that can be processed digitally to expedite analysis of biological samples. This is the case for X-ray micro Computed Tomography (µCT) scanning (Masschaele et al., 2007; Cnudde et al., 2011), Light Sheet (based) Fluorescence Microscopy (LSFM) (Santi, 2011; Buytaert et al., 2012), Optical Projection Tomography (OPT) (Sharpe et al., 2002), standard and phase-contrast synchrotron X-ray imaging (Betz et al., 2007; Boistel et al., 2011) and magnetic resonance imaging (MRI) (Tyszka et al., 2005; Pohlmann et al., 2007). LSFM uses laser light sheets to illuminate a fluorescent and transparent sample, while OPT uses light rays to image a transparent sample. Synchrotron X-ray imaging uses electromagnetic radiation. MRI uses magnetic fields and radio waves to image the specimen and detects differences in water content and distribution of fluids in the soft tissues near or around bones in a sample. µCT-scanning, which uses X-rays to create cross-sections in a sample, is based on contrast differences in X-ray absorption (i.e. the attenuation contrast between different tissues). All techniques mentioned

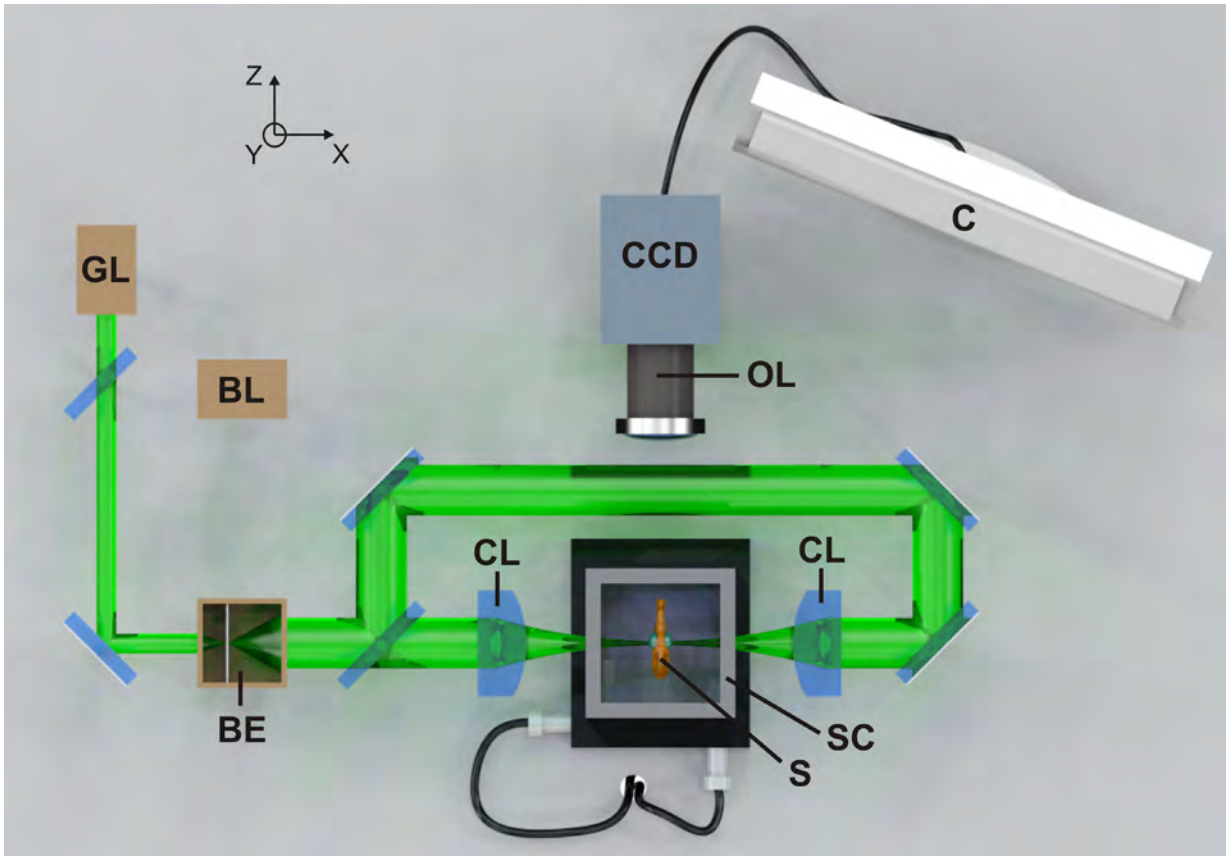


Fig. 1. – Top view of a three-dimensional representation of the (HR)-OPFOS set-up. In this case the green laser (GL) is active, while the blue laser (BL) is not active. The laser light first passes through a beam expander (BE) to increase the diameter of the input beam in the Y and Z dimensions and subsequently passes a cylindrical lens (CL) to focus the beam into a thin light bundle (reducing the beam in the Z-dimension) before it transverses the transparent and fluorescent sample. The sample is immersed in Spalteholz fluid and lies in a sample chamber (SC). The sample emits fluorescent light, which is projected on a CCD camera with an objective lens (OL). The light forms images of the optical sections that are displayed on a computer (C).

above are useful to display the biological specimens in three dimensions, despite the specific limitations of each one. An important constraint of non-destructive approaches is the initial investment in the required infrastructure (e.g. synchrotron facilities). Other techniques suffer from resolution limitations or lack the capability to discriminate between different tissue types, while others fail in penetrating organ systems of interest to a sufficient depth, hence limiting the size range of samples that can be studied.

In 1993, a new alternative microscopic and tomographic approach, the Orthogonal-Plane Fluorescence Optical Sectioning microscopy (OPFOS) emerged in order to simplify the generation of 3D models of complex structures and to make quantitative measurements of the mammalian cochlea (VOIE et al., 1993). OPFOS is a whole-volume imaging method that creates virtual sections by projecting a thin sheet of laser light through the fluorescent and transparent sample (Fig. 1). The sample is excited by the laser light and the fluorescent and autofluorescent light is detected orthogonally with an objective and recorded by a camera. The sample is moved along the Z-axis through the laser light sheet and recorded at different depths. In this way, virtual sections are compiled throughout the specimen and used to generate 3D reconstructions.

OPFOS belongs to a whole new microscopy field, designated Light Sheet based Fluorescence Microscopy (LSFM) (SANTI, 2011; BUYTAERT et al., 2012), including many other implementations such as Selective Plane Illumination Microscopy (SPIM) (HUISKEN et al., 2004), high resolution (HR-) OPFOS (BUYTAERT & DIRCKX, 2007), Ultramicroscopy (DODT et al., 2007) and Thin-Sheet Laser Imaging Microscopy (TSLIM) (SANTI et al., 2009). SPIM has already been used in a study of *in vivo* imaging of the embryogenesis of *Drosophila melanogaster* and muscles in Medaka fishes (HUISKEN et al., 2004). Of the dozens of LSFM implementations, however, only Ultramicroscopy, TSLIM and (HR-)OPFOS (BUYTAERT & DIRCKX, 2007) are capable of imaging macroscopic samples (size range of tens of millimeters). For instance, membranes, suspensory tissues and bones of the middle ear and inner ear were investigated with OPFOS (VOIE, 2002; HOFMAN et al., 2008; BUYTAERT & DIRCKX, 2009; HOFMAN et al., 2009; BUYTAERT et al., 2010; BUYTAERT et al., 2011) and TSLIM (SANTI et al., 2009). Whole mouse embryos and particularly their brains have also been studied with Ultramicroscopy (BECKER et al., 2008). Despite the great utility of LSFM implementations, it has not yet found its way into many fields of biology.

A qualitative comparison between 3D imaging of the musculoskeletal system in a vertebrate head using a completely destructive, largely manual technique (SHS) and a much less destructive, largely digital technique (OPFOS) was conducted in this study. The main purpose of this investigation was to give an overview of the advantages and disadvantages of the use of OPFOS compared to SHS in generating a 3D reconstruction of a similar specimen. In the present study, OPFOS has been applied for the first time on the model organism *Xenopus laevis*. A tadpole of *Xenopus* is a suitable model organism as it is small with a semi-transparent skin that allows a non-invasive investigation of the anatomy of all internal structures. We hypothesized that the 3D models would be more accurate using OPFOS instead of histological sections, since no alignment of the images is necessary and no tissue distortion is induced by mechanical slicing. In addition, shrinking of organs, induced by elaborate specimen preparation, is a well-known phenomenon with both OPFOS (BUYTAERT et al., 2012) and histological sectioning (LANE & RALIS, 1983; HOFMAN et al., 2009). However, we expected that the shrinking effects would be uniform and thus largely negligible for the interpretation of the 3D anatomy.

With the OPFOS method, both omnidirectional fluorescence and autofluorescence can be recorded. Many tissue components exhibit autofluorescence, which is a natural light

emission. The molecules responsible for this spontaneous tissue fluorescence include flavins, nicotinamide adenine dinucleotide (NADH), lipofuscin, elastin, collagen and porphyrins (ANDERSSON et al., 1998; BILLINTON & KNIGHT, 2001). Therefore, we expected to find differences between tissues with distinct autofluorescence values. We also expected that discrimination between different tissue types and organs may be done in an automated way, since different tissue types and organs may display different intensities of autofluorescence. Finally, we wanted to test the potential of this method for discrimination between different structures of the same tissue type (e.g. muscle-muscle or skeleton-skeleton).

## MATERIALS AND METHODS

### Specimen preparation

Three African clawed tadpoles (*Xenopus laevis*) were used, one in stage 46 for histological sectioning and light microscopy, and two in stage 47 for OPFOS microscopy. This difference in developmental stage is too small to hinder the comparison and should not be considered as an explicative factor for the observed differences. These organisms were macroscopic, which means that they could be perceived by the naked eye, a requirement for the set-up of the OPFOS used in this study. The *Xenopus* larvae were euthanized by MS222 (Sigma E10521) and fixed by immersion in 4% paraformaldehyde. Thereafter, the specimens were processed for dehydration through an ethanol series (30%, 50%, 70% and 100%).

### Procedure for SHS

Once placed in 100% ethanol, the specimen was processed for embedding in Technovit 7100 (Heraeus Kulzer Wehrheim, Germany). Serial histological cross-sections of 5µm (slice thickness) were cut using a Leica Polycut SM 2500 microtome. Each section was mounted on a glass slide, stained with toluidine blue and covered. A total of 237 histological sections were used for further investigation. Digital images of those sections were taken using a Colorview8 CCD camera (SIS) placed on a Reichert Polyvar microscope, managed by the software program analySIS 5.0. The pictures of the sections were loaded in the 3D graphics software Amira 5.0 - Visage Imaging (64-version, Mercury Computer Systems) and aligned semi-automatically. Different anatomical structures were identified, but only relevant structures (i.e. muscles and cartilaginous elements of the cranium) were segmented (isolated digitally) manually. A virtual 3D-reconstruction was then generated based on those segmented structures.

### Procedure for OPFOS

The OPFOS technique required an elaborate specimen preparation (VOIE, 2002; BUYTAERT & DIRCKX, 2009). Between the fixation step and the dehydration step, the tadpoles needed to be bleached. Tadpoles have a pigmented skin, hence, they were bleached in a 5% hydrogen peroxide solution ($H_2O_2$) for one hour. After that, they were cleared and optionally stained with a fluorescent dye. The clearing process involved placing the specimens in a graded series of Spalteholz fluid (25%, 50%, 75%, 100% and 100%), which consists of five volumes of methylsalicylate (oil of wintergreen) and three volumes of benzyl benzoate. This method matches the refraction index of the entire sample to that of the fluid, making the entire sample optically transparent. In this study, a blue laser light was used for the illumination of the sample. The sample emitted autofluorescence at a wavelength of 488 nm.

With OPFOS, a FOculus FO531SB grayscale camera (NET GmbH) was used, equipped with a combined QIOPTIC Optem Zoom 125C optical lens system comprising a 1x Qioptiq art 30-13-10 (15 mm fine focus module), a 1x Qioptiq art 30-61-40 (zoom module with detents, no iris) and a 1x Qioptiq art 29-90-73 (1.5x TV tube). Pictures with pixelsize 2.3 x 2.3 µm were taken of the illuminated plane every 3µm and stored. The collected image data had a bit-depth of 12-bit (4096 gray-scale values). Up to 567 images

were loaded in the 3D graphics software program Amira 5.0 - Visage Imaging (64-bit version, Computer Systems Mercury). For comparison, the anatomical structures labeled for SHS were identified and segmented manually based on gray-scale values. 3D reconstructions of the labeled structures were then generated.

### Advantages and disadvantages of shs

The SHS technique provides high resolution images (the only limitation is the resolution of the optics of the light microscope) and results in detailed 3D reconstructions. The major structures can be discerned down to the cellular level, where individual cells can be seen clearly. Sometimes, even details down to the subcellular level can be identified. The high quality imagery is highlighted in Figure 2 (A-B). One of the advantages of SHS is that the sections are always accessible. Later, it is possible to go back to the samples and look for specific details. Tissue types such as muscle tissue, skeletal tissue and neural tissue can be discerned very easily based on the staining of the sections (Figs 2 and 3). In this study, the muscles as well as the structures of the nervous system are stained entirely, resulting in blue structures of variable blue gradients discernible from each other. On the contrary, the cartilaginous skeletal structures are not stained completely, as only the extracellular matrix, the chondrocytes and nuclei are stained blue (Fig. 3C). The lacunae, being occupied by the chondrocytes (which are unfortunately not well preserved during fixation), are large cavities in the matrix, appearing white after the sections have been stained. With the light microscopic histology technique, bones as well as cartilage can be visualized simultaneously. Since the animals in our study were too young, bone was not present, but the technique would allow visualization of cartilage and bone.

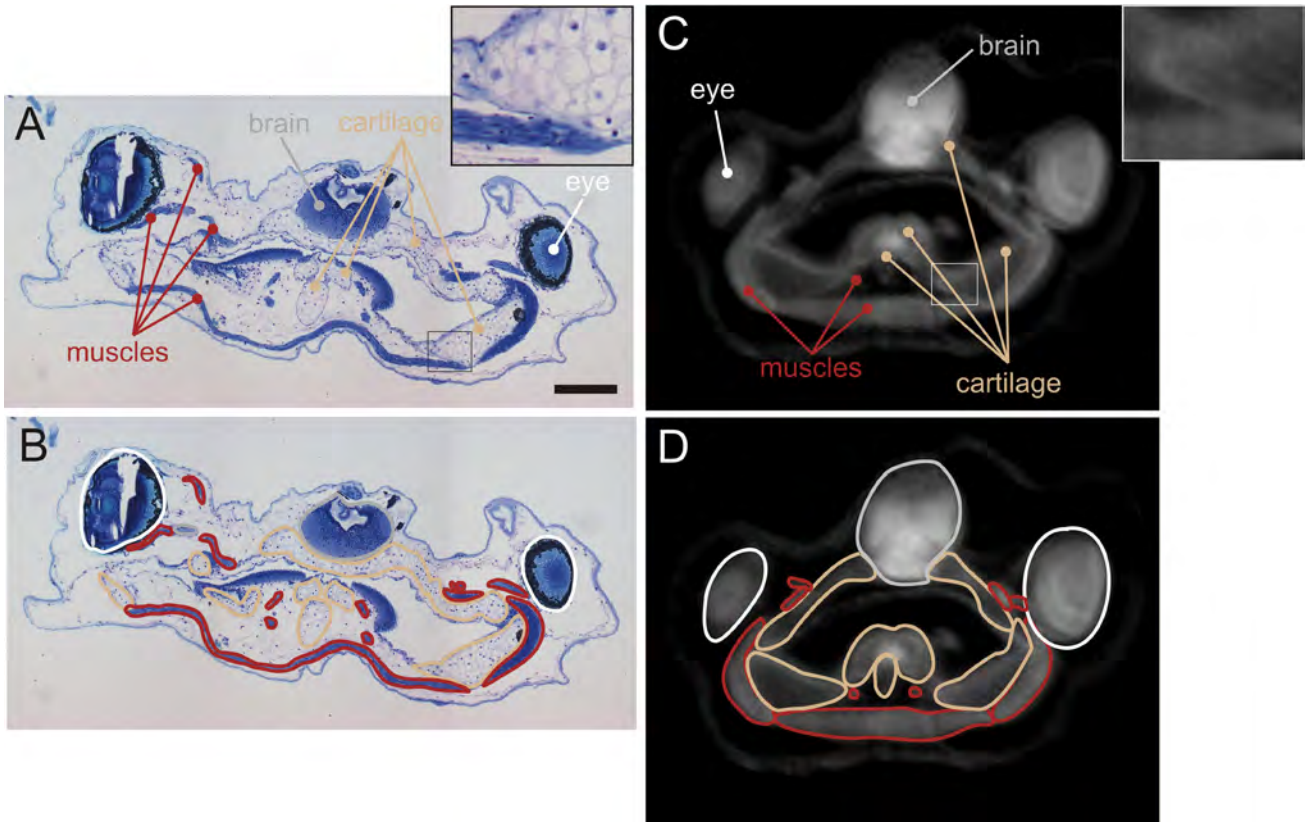


Fig. 2. – Cross-sections of the head of *Xenopus laevis* tadpoles generated with (A,B) histological sectioning and (C,D) OPFOS. The colored lines on panels (B,D) demonstrate the labeling of the different structures before 3D reconstruction. Magnification of some cartilage and muscle tissues is shown in the upper right insets of panels (A,C). Scale bar: 200 µm.

The SHS technique is, however, labour-intensive and particularly time-consuming. First, the specimens need to be fixed, decalcified (when they contain mineralized bone) and dehydrated. After this, the specimens have to be sectioned mechanically (one cutting direction) in very thin slices, and are thus destroyed. For this reason, the sample cannot be used more than once or used for other purposes. Next, pictures of the sections have to be taken and saved individually. Consequently, image processing and registration are necessary and quite difficult. Accordingly, not every section is included in a traditional reconstruction, because this would increase the workload to take pictures of every slice at a high resolution. This also means that every virtual section that diverges in orientation from the cutting plane (i.e. along the Z-axis) will provide fewer details. Thereafter, the spatial alignment of the slices into a 3D dataset has to be done manually and artefacts can be introduced through imperfect aligning. The latter are partially induced by mechanical deformation of the sections as a result of sectioning and subsequent stretching (Fig. 3 A-B), and partially because there are often no adequate reference points for the alignment of the sections. Because of misalignment and distortion in individual slices, the final 3D reconstruction is less smooth, giving it an impression of having a rough surface (Fig. 4). The position of the individual sections can still be seen on the 3D reconstruction, which can be due to manual alignment, deformation caused by cutting the sample, and the smaller number

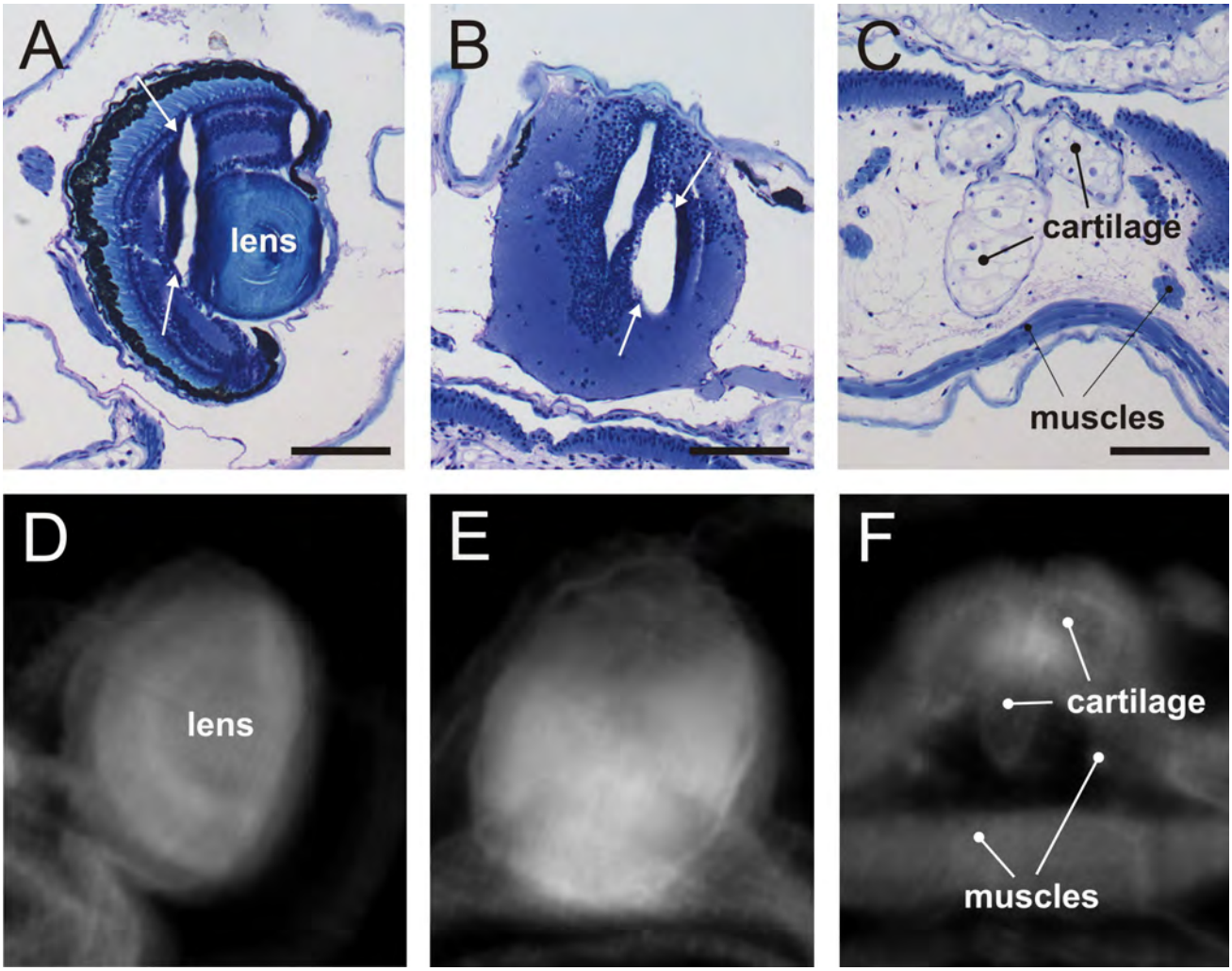


Fig. 3. – Comparison between the two techniques: (A-C) histological sectioning and (D-F) OPFOS. Cross-sections at the level of (A,D) eye; (B,E) brain; (C,F) cartilage and muscles. Arrows show artifacts in the histological sections due to specimen preparation. Scale bar: 100 µm.

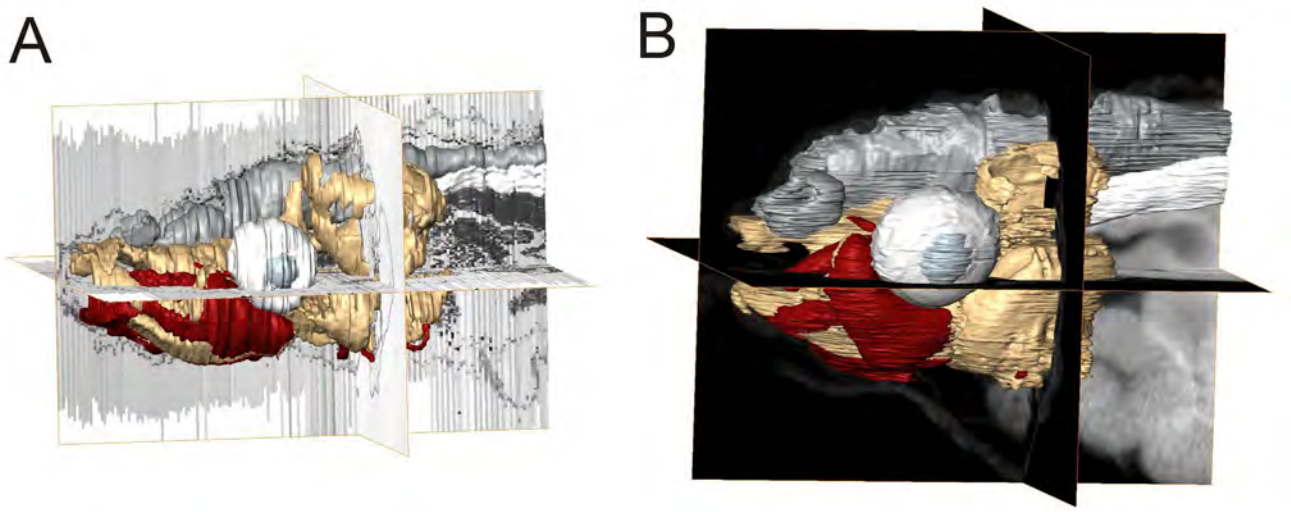


Fig. 4. – Lateral view of two 3D reconstructions of *Xenopus laevis* head with cross sections in three orientations generated with (A) histological sectioning and (B) OPFOS. Different colors represent different tissue types (beige: cartilage, red: muscles, grey: brain and nervous system).

of sections used for the reconstruction (as only a fraction of all available sections is used). In the latter case, the distance between two successive sections is larger than the X- and Y-voxel size. Moreover, artefacts such as shape distortions of tissues are unavoidable. In this study, for example, the reconstructed specimen seems to be dorsoventrally flattened and especially the brain appears to be collapsed (Fig. 4). This kind of distortion, which may be due to mechanical slicing (knife orientation), cannot be undone. Some distortions can sometimes be partially undone by supplementary manual adaptations, such as interpolating the tissue segmentations on the sections just before and just after the distorted section. Finally, light microscopic histology, as applied in this study, relied on the use of expensive instruments including a microtome, a

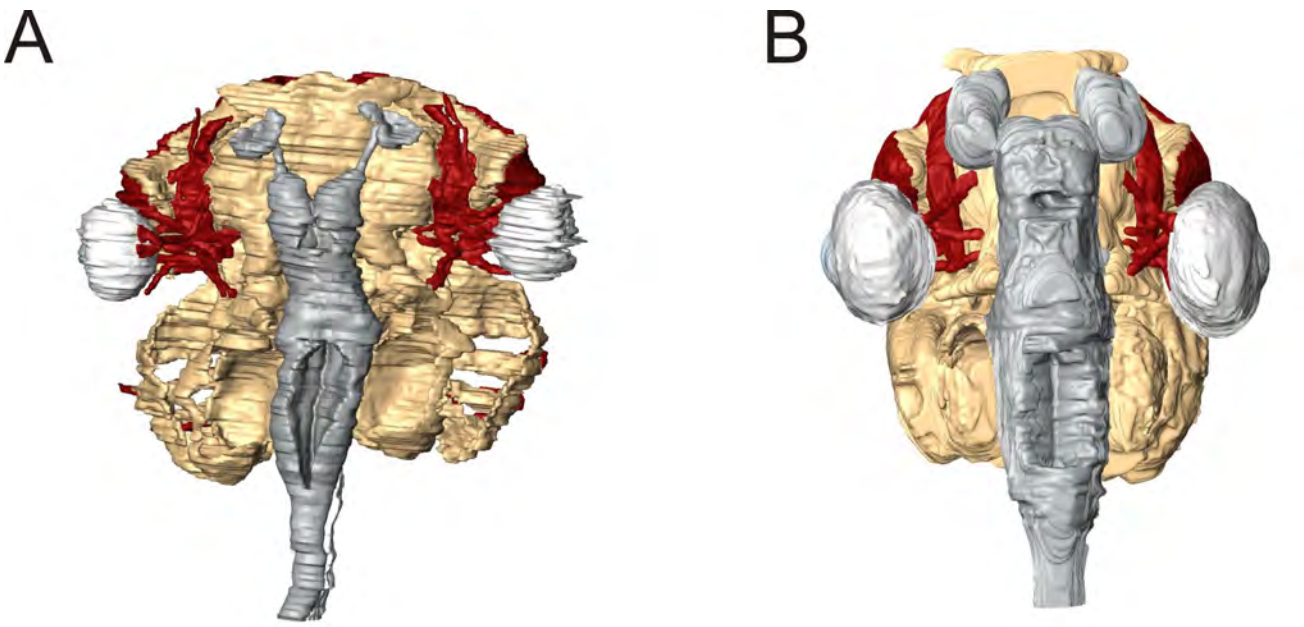


Fig. 5. – Dorsal side of 3D models of *Xenopus laevis* heads, using (A) histological sectioning and (B) OPFOS.

histokinette, an automatic glass coverslipper, a stereomicroscope and a camera.

### Advantages and disadvantages of opfos

The virtual sectioning and imaging are performed quickly and in real-time, and are consequently less time-consuming. There are no registration difficulties as image registration between successive sections is done automatically. The data do not need to be aligned, since it is a whole-mount and semi-destructive imaging technique, where the data are already aligned in the image database. This advantage leads to a smooth 3D reconstruction. Moreover, the images obtained with the OPFOS technique have a good resolution. The highest axial resolution (Z-dimension) that can be obtained with high resolution (HR-)OPFOS is 2µm (Buytaert & Dirckx, 2007). Lateral (X and Y) dimensions can even go below micron level. In the sections in this study, an axial resolution of about 5 µm was obtained. Region-of-interest imaging, which is the imaging of only a substructure of the object, can be performed with OPFOS. This may deliver detailed microscopic and even histological information (at cellular level). However, since ours are preliminary data, no region-of-interest has been imaged. This approach is suited to visualization of bony structures (after decalcification) and soft tissues (such as muscles and nerves) at the same time. The different tissue types, such as muscle tissue and skeletal elements, can be discriminated very easily by their own tissue-specific gray levels. The obtained images are thus composed of different grayscales (Figs 2 and 3). Skeletal structures are darkest on the OPFOS images, reflecting the lowest levels of autofluorescence while the muscles and nervous system are the brightest structures. The boundary between two adjacent structures, having different tissue types, can be discerned based on those distinct grayscales and their mutual topography. This allows manual tracing (Fig. 2D) and the generation of a 3D reconstruction (Figs 4B and 5B) of the individual structures on the image stacks acquired by OPFOS. If two adjacent structures have the same tissue type (e.g. muscle-muscle) and thus the same gray levels, however, prudence and knowledge on the anatomy are called for in the interpretation of their boundary depending on the quality of the images.

OPFOS microscopy is a whole-volume imaging method that does not destruct or touch the specimen, since a laser light sheet is used to generate the sections. Therefore, several 2D images series can be recorded with a different slicing orientation and one specimen can be used multiple times. Moreover, all structures remain in their natural position and there are no artefacts due to an imperfect alignment of the optical sections. Furthermore, functional staining with a fluorescent dye (such as Rhodamine B) can be applied in order to obtain stronger fluorescence and to better discriminate between tissue types. Even multiple dyes can be incorporated to stain different tissue types, though each requires its own specific laser light source that emits light of a specific wavelength. Another advantage of OPFOS microscopy in this study is that it utilised relatively inexpensive components, including a sample chamber, a light source, a CCD camera and optics. This makes the OPFOS accessible to a larger group of researchers.

The preparation of the specimen is, however, extensive (Voie, 2002; Buytaert & Dirckx, 2009). An elaborate procedure must be followed: fixation, bleaching, EDTA decalcification, dehydratation and refractive index matching or clearing. The bleaching with hydrogen peroxide and decalcification with EDTA are optional and irreversible, although they are required when pigmented tissue and calcium content are present. In the first specimen that was scanned with OPFOS, the light was blocked or attenuated in some parts of the sample due to remaining pigmentation. Consequently, the OPFOS images were reduced in quality as typical striped shadows (black masses) (Buytaert & Dirckx, 2009; Buytaert et al., 2011; Buytaert et al., 2012) were generated in the images (Fig. 6). This is caused when the illumination sheet originates from only one side of the image. When the laser

sheet then encounters a pigment, the light and thus also the tissues behind this pigment are attenuated, resulting in shadow artifacts. Those black masses make it difficult to segment the missing structures prior to 3D reconstructing. To solve that problem, the method was refined during the experiment by adding an additional bleaching step. Two-sided illumination (Fig. 1) partially solves this problem too. However, this requires a more difficult calibration and aligning of the set-up. In this study, the two-sided illumination unfortunately resulted in blurred images (Figs 2 and 3). Sharper images would reduce the time of segmentation of each structure and even allow this segmentation in a semi-automated or automated way. Such sharper images may be obtained by a thinner laser light plane, a lens objective of a better quality, less vibrations and a higher resolution of the camera.

Fig. 6. – Virtual section of the head of a *Xenopus laevis* tadpole generated with OPFOS. At the level of the arrows, where a structure is expected, a black region is noticed. This is due to pigmentation on the tadpole's skin, resulting in blocked or attenuated light.

Other steps in the preparation process are decalcification and clearing. Calcium, which is the main component of bone, scatters light strongly. In this study, the specimens had no bony structures, so no decalcification was necessary. Clearing of specimens leads to their irreversible transparency, preventing, for example, any histological post-processing. After specimen preparation and imaging, anatomical structures on hundreds of sections have to be segmented manually – a time-consuming procedure.

## Summary of similarities and differences in methods

Both OPFOS and SHS techniques required elaborate specimen preparation. Refractive index matching and bleaching were extra steps in the preparation process of OPFOS over histology. The OPFOS set-up was very sensitive to remaining eye and skin pigmentation, necessitating bleaching of the tadpole in order to make pigmented tissue transparent. Interestingly, the bleaching step is not necessary for specimens that become sufficiently transparent during the specimen preparation (e.g. young seahorses). Moreover, the real-time virtual sectioning of OPFOS makes it an ideal tool for fast screening of macroscopic animals (size range of tens of millimeters), while SHS requires manual, labour-intensive and destructive sectioning. As the OPFOS technique is semi-destructive, the specimens can, however, not be used again for histological sectioning or immunohistochemical analysis afterwards. This is actually possible with samples scanned with, for example, µCT. OPFOS-like techniques nevertheless suffer from the fact that the specimens are made irreversibly transparent. This is also the case with OPT (Optical Projection Tomography), but not with µCT and MRI, where the specimens can be reused for other purposes. An advantage of OPFOS over OPT and µCT is that the cross sectional area is immediately registered, while with OPT and µCT the data first needs back-

projection calculations to recreate the original object (Kak & Slaney, 1988).

2D sections with microscopic details were generated with SHS, while with OFPOS fewer details were revealed. Even in 3D, SHS showed more details than OPFOS. Particular structures can be visualized with SHS using a staining protocol comprising several dyes. Some staining protocols are capable of staining cellular structures, such as myelin, muscle striations, etc. In the same way, functional staining has the potential to be applied to OPFOS.

OPFOS does have the simplicity of allowing discrimination between tissue types of different organ systems thanks to their distinctly different grayscales because it relies on (native) tissue contrast. On the other hand, SHS depends on histological staining, where as a result even the same tissue might have a different color intensity from section to section. On the virtual sections of OPFOS, differences between tissues with distinct autofluorescence values were found as expected. However, a problem arises using OPFOS where organ systems having similar tissue types lie side by side (i.e. muscle-muscle) and have similar grayscale values. The combination of blurred images and similar tissue types resulted in images that were of insufficient quality to generate an accurate 3D model. In this case, the boundary between different structures could not always be accurately determined. This limitation prevented automatic discrimination of different tissue types. The histological sections were therefore very useful for labeling the virtual sections of OPFOS.

One of the biggest advantages of both OPFOS and SHS is their ability to visualize bony structures and soft tissues simultaneously. This is also an advantage for MRI (Tyszka et al., 2005; Pohlmann et al., 2007) and OPT. Using simple staining techniques, including contrast agents such as osmium tetroxide ($OsO_4$) or phosphotungstic acid (PTA), µCT can provide high-contrast 3D imaging of soft tissues as well (Johnson et al., 2006; Metscher, 2009a, b).

The 3D reconstructions showed that the OPFOS provided a more naturally-shaped 3D model than the one based on histological sections. With OPFOS, all structures remained in their natural position, while in the reconstructions generated with histological sections small irregularities as well as an apparent flattening of the 3D model were discernible (Fig. 4). Using SHS, all sections needed to be aligned manually, which in practice could not be done without artefacts. Extra compensations were also necessary for distortions due to the preparation procedure of the sections. Another kind of distortion, which occurred in both SHS and OPFOS, is shrinking. Shrinking of tissues in all three dimensions was previously reported for OPFOS (Voie, 2002; Hofman et al., 2008; Buytaert et al., 2011). This shrinking is induced by the elaborate specimen preparation (fixation, decalcification and dehydration). On the other hand, shrinking is also well-known to occur in SHS as the preparation also includes fixation, (decalcification) and dehydration (Lane & Ralis, 1983). Therefore, deformations due to specimen preparation were expected with both protocols. Comparing both 3D reconstructions, we noticed that our OPFOS model looked different from the SHS model as if some structures have shrunk in the SHS model (Fig. 5). Most obvious were the smaller eyes and nasal capsules, indicating a more invasive influence of the SHS procedure on the specimen. In this way, our hypothesis that a more accurate 3D model is obtained with the OPFOS technique can thus be confirmed.

The highest resolution for macroscopic specimens, which is about 0.2µm, is provided by standard histological sections. In our study, the 2D histological sections provided a better resolution than OPFOS. The attainable resolution with (HR-)OPFOS is around 2µm for macroscopic specimens (Buytaert & Dirckx, 2007), but in this study it was 5µm. It is, however, to be expected that SHS will always slightly outperform OPFOS on resolution of 2D section images. Other LSFM methods, however, can achieve submicron resolution on microscopic samples. Regarding other modern automated techniques, µCT can

currently easily reach a resolution of 1µm (MASSCHAELE et al., 2007) and sometimes even resolutions of 700nm (CNUDDE et al., 2011). Real-time synchrotron X-ray phase-contrast imaging (SOCHA et al., 2007) is capable of reaching a resolution of about 1µm, but there is an important trade-off between the spatial resolution and the detrimental effects on the specimen. OPFOS has conversely a higher resolution than OPT (Optical Projection Tomography), which is limited to a resolution of 5 to 10µm (SHARPE et al., 2002), and MRI, which attains a lower resolution of about 25µm (SCHNEIDER et al., 2003b, c; SCHNEIDER et al., 2003a).

Finally, the infrastructure cost for the basic implementation of OPFOS microscopy used in this study was estimated to be more than 15 times lower than that for SHS. Newer and better OPFOS devices are still cheaper than SHS. Other expensive imaging techniques are for example the µCT scanning, MRI and synchrotron X-ray imaging (SANTI, 2011).

## FUTURE ADVANCES AND CONCLUSIONS

From our preliminary study, we can conclude that OPFOS tomography is a good technique for the investigation of musculoskeletal systems in macroscopic specimens, as shown here for tadpoles. As expected, SHS achieved a better spatial resolution, which is important to see microscopic details and obtain essential information. Unfortunately, this could not be obtained with the basic implementation of the OPFOS technique used in this study. Several improvements to the (HR-)OPFOS tomography have already been made (BUYTAERT et al., 2011) and the LSFM field is still evolving. Therefore, a more elaborate comparison that includes more advanced OPFOS versions would be useful to get a better overview of the advantages and disadvantages.

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# Burrow morphology of three species of fiddler crab (*Uca*) along the coast of Pakistan

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ABSTRACT. Burrowing by crabs is an important component of their functional role in mangrove biotopes. The Fiddler crab (*Uca*) is one of the more conspicuous burrowing organisms in the mangrove areas of Pakistan. To evaluate interspecific differences in burrowing behaviour between three species of *Uca* (*U. annulipes, U. chlorophthalmus* and *U. sindensis*), we compared vegetation cover, sediment composition and burrow morphology by using plaster of Paris casts. Five burrow morphology characters were measured (burrow number, depth, length, volume, and diameter of the burrow openings). Nearly all the morphological characters of the burrows differed significantly between species. The burrow morphology variations were correlated with the tidal level (distance from the water mark during low tide), porosity, percent organic matter, vegetation cover and structure of the sediments. The species-specific differences in the burrows cautions against generalizing regarding the functional role of fiddler crabs along the coast of Pakistan.

KEY WORDS: burrows, fiddler crab, ecosystem engineer, biotopes, burrow cast, tidal height.

## INTRODUCTION

Fiddler crabs are semi-terrestrial organisms that may influence the sediment structure and other living communities mainly through their burrowing and feeding activities (Teal, 1958). Fiddler crabs construct their burrows actively in the intertidal zone from coarse beach sand to fine clay-rich marshy mud during low tide. The digging of burrows begins when the crabs are very small (Hyman, 1922; Herrnkind, 1968). The digging behavior of fiddler crabs has been described by various authors (Altevogt, 1955; Crane, 1975). These crabs are known to adjust their burrowing activities to a variety of conditions, such as stem density, root mat density, substratum, water, ground temperature, tidal periodicity, reproductive activity, threat by potential predators, seasons and mate display activities (Ringold, 1979; Bertness, 1985; Genoni, 1991). Faunally-mediated disturbances of the physical, chemical and biological structure of the sediment are known as 'bioturbation'. The bioturbation and fecal pellet production of fiddler crabs in mangroves have been described (Warren & Underwood, 1986; Dye & Lasiak, 1986; Skov et al., 2002; Bartolini et al., 2011). They result in increased growth of the cord grass, *Spartina alterniflora*, in salt marshes (Montague, 1980; 1984; Bertness, 1985). The resulting biogenic structures and the engineering processes involved in constructing and maintaining burrows generate complex interactions associated with trophic webs (Reise, 2002; Kristensen, 2008).

Burrows are important to fiddler crabs for a number of functions, allowing them to adopt a semi-terrestrial existence and avoid environmental stresses by behavioral means (Atkinson & Taylor, 1988). Fiddler crabs excavate burrows in the sediment, to avoid excessive wave action and obtain relief from hot and cold ambient temperatures and desiccation

(Hyatt & Salmon, 1979; Bertness & Miller, 1984; Lim & Diong, 2003). Burrows provide a refuge from both aerial and terrestrial predators during exposed periods and from aquatic predators during flooding, water for physiological needs, and also sites for molting and reproduction (Hyatt & Salmon, 1979; Katz, 1980; Christy, 1982; Thurman, 1984; Bertness & Miller, 1984; Genoni, 1991; Lim & Diong, 2003; Lim, 2006; Milner et al., 2010).

The architecture of the burrows plays an important ecological role in the life history of fiddler crabs as they are semi-terrestrial and active at low tide, returning to their burrows at high tide. Since the burrow is a place of refuge from predators, its structure must be advantageous to the crab when it is necessary to make a hasty retreat when pursued or threatened. Especially in anoxic sediments burrows have greater ecological significance as they help to improve the aeration of the sediments. Burrow morphology has been described by using plaster of Paris, rubber, or epoxy resin casts. General burrow morphology varies in diameter, depth, volume, and angle of the burrow with respect to the shoreline. General burrow design has been studied in several species of the fiddler crab including *Uca pugilator* (Dembowski, 1926; Christy, 1982), *U. rapax* (Genoni, 1991), *U. pugnax* (Gray, 1942; Katz, 1980; Bertness & Miller, 1984), *U. panacea* (currently synonymous with *U. pugnax* see Powers, 1975), *U. longisignalis, U. spinicarpa, U. vocator, U. subcylindrica* (Thurman, 1984), *U. tangeri* (Wolfrath, 1992), *U. annulipes* and *U. vocans* (Lim & Diong, 2003; Lim, 2006).

We hypothesized that the morphology of the burrows would vary between the three species of fiddler crabs, *U. annulipes, U. sindensis* and *U. chlorophthalmus,* as they prefer biotopes varying in tidal height, vegetation and sediment composition. There has been no previous comparative study of the burrow morphology of fiddler crabs along the coast of Pakistan. In the present study, we investigated differences in structural morphology and complexity of three species of fiddler crab burrows in their respective biotopes to identify the relationship of burrow morphology of *Uca* species according to their habitat found along the coast of Pakistan.

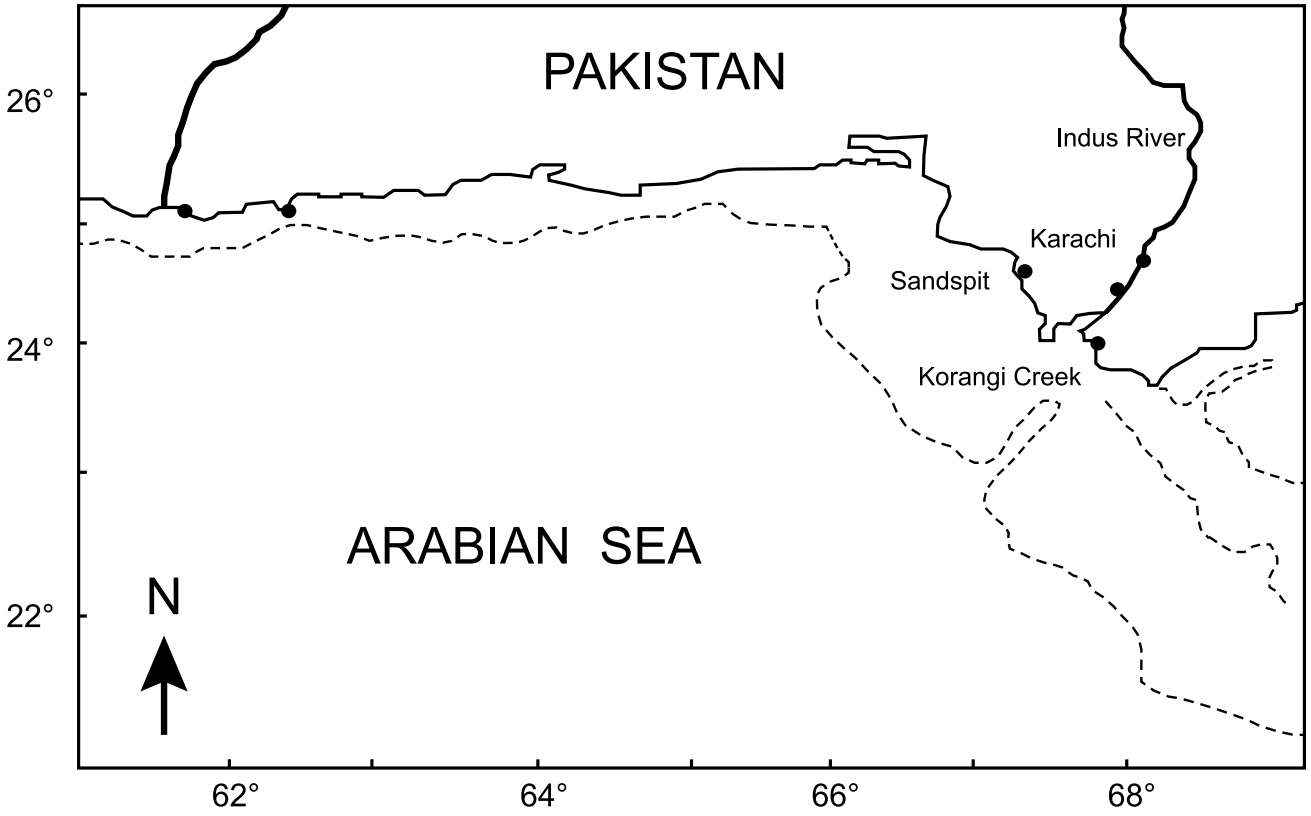


Fig. 1. – Coastline map of Pakistan showing the study sites i.e. Sandspit and Korangi creek mangrove areas.

## MATERIAL AND METHODS

### Study sites

Two sites, Sandspit and Korangi creek, were selected for the study based on the presence of separate patches of three species of fiddler crabs (Fig. 1). These sites differed markedly in terms of vegetation, tidal height and sediment properties, related to the habitat preferences of the respective species.

### Site 1

The Sandspit backwaters mangrove area is located at (24°50'N, 66°56'E) south west of Karachi. The backwater is connected to the Arabian Sea through the Manora Channel. Sandspit beach is divided by a strip of dry land, with mud flats and mangrove vegetation found on the northern side. and sandy coast on its south. The dense vegetation comprises a monospecific stand of the mangrove species *Avicennia marina.*

### Site 2

The second study site was located (24°79'N, 67°20'E) in the Korangi creek mangrove area near the salt works in the fishing village of Ibrahim Hyderi. The northernmost creeks of the Indus Delta are the Korangi and Phitti creeks of which Korangi creek is 12 km from Karachi Harbour and 9 km from Quaidabad. Korangi creek is connected at its northeastern end with Phitti and Kadiro creeks, while at its southwestern end, it connects with open sea and with Gizri creek, and the study area is bounded by extensive mangrove vegetation of *A. marina*.

### Field Methodology

The identification of the burrow is an important issue in the study of burrow morphology of *Uca* species with sympatric distribution. For example, at a few sites *U. sindensis* coexists with *U. chlorophthalmus* whereas at other sites *U. chlorophthalmus* coexists with *U. annulipes*, and it was quite difficult to determine which burrow belonged to which crab species without identifying a pure distribution patch of each species. In this study the burrow cast data for *U. annulipes* and *U. chlorophthalmus* were collected from the two different stations of Sandspit and data for *U. sindensis* were collected from Korangi creek mangrove area. The plaster casts were found to be perfect for measuring the diameter, total length, total depth and volume of the burrows.

Burrow casts were made for the three species *viz*: *U. sindensis, U. chlorophthalmus* and *U. annulipes.* At selected sites, counts of open burrows were first conducted as these indicate the number of burrows present beneath the surface. The burrow-opening density within the distribution area of each species was estimated by randomly locating 0.25 $m^2$ quadrats (6 replicates). The total number of open burrows within each quadrat was counted. In each quadrat three or four burrows were randomly selected for casts.

Aqueous solution of plaster of Paris was poured into the selected crab burrows with the help of a syringe until the burrows were completely filled, then allowed to dry for 30 to 60 minutes (WARBURG & SHUCHMAN, 1978). If the crab emerged during this process, it was hand picked and placed in a marked poly bag for further analyses. The casts were then carefully dug up by hand, or with a spade in the case of hard substratum, then cleaned to remove as much sediment as possible from the surface of the cast. Each cast was separately placed in pre-marked poly bags and brought back to the laboratory for further analyses. After the casts were dug out, the area within each quadrat was excavated to a depth of 30 cm and the crabs were collected to calculate the relationship between the density of crabs and crab burrows. Three sediment cores (up to 30 cm in depth) were also taken adjacent to the casting area for each quadrat replicate, to analyse sediment properties. For each species, the different diameters of burrow openings (n = 45 at least) were also analyzed.

Crabs were caught within the burrows and at the mouths of their burrows. Burrow diameters were measured with vernier callipers and the following parameters of crabs recorded: sex, carapace width, carapace length (CL). The size (carapace length) of resident fiddler crabs can be determined for each species by using a linear equation expressing the relationship between CL and BD (burrow diameter). As a crab always enters its burrow sideways, (LIM & DIONG, 2003) carapace lengths have been used to study the relationship between resident crabs and their burrow diameters. The burrow diameter and crab length relationship was evaluated by the best fit regression line

$$\text{Carapace length (CL)} = a + b\,(\text{burrow diameter (BD)})$$

where a and b are coefficients to be estimated by the model. Data for males and females were pooled, as we observed no differences for the above parameters between the sexes during preliminary regression analysis.

## LABORATORY ANALYSES

In the laboratory, sediment properties (percentage of organic matter and grain size) were analyzed. Percentage of organic matter was determined by the loss in mass after combustion in a muffle furnace, following SAHER & QURESHI (2010). Briefly, a 20-50 g sample of dry sediment was placed in a pre-weighed crucible, covered with a lid and combusted at 450 °C for 3 hrs. Grain size was analyzed by dry sieving methodology following FOLK (1974).

The morphology, size and structure of fiddler crab burrows were determined (Fig. 2). The parameters measured for each burrow cast were: burrow diameter (BD), total burrow length (TBL), total burrow depth (TBD), and burrow volume (BV). Volume was determined by weighing each cast (± 0.1g) and dividing the weight of the burrow cast by the density (2.2 g $cm^{-3}$) of plaster of Paris (CHAN et al., 2006). Only data for complete burrow casts were used for analyses.

### Statistical analyses

Data for all burrow parameters studied and estimated were statistically analysed using Minitab (Version 15.0). A t-test was employed under the null hypothesis that the expected burrow densities were equal to the number of crabs. One-way analyses of variance (ANOVA) were used to test for differences between the species for density, total length, total depth, diameter and volume of burrows. Differences were accepted as significant at alpha = 0.05 for statistical analyses. Tukey's pairwise comparison tests were carried out when main effect ANOVA tests were significant for the burrow cast variables of the three species of *Uca* to relate variables to habitat. Pearson correlation coefficients were calculated to determine the relationship between burrow densities and sediment properties (percent organic and grain size).

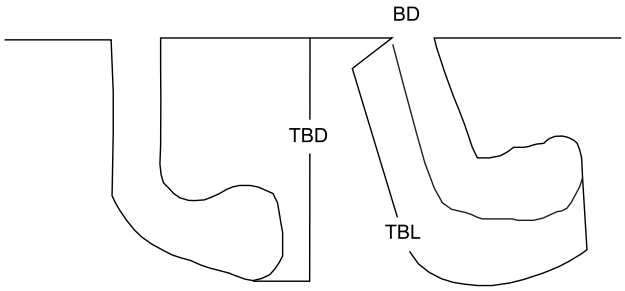


Fig. 2. – Burrow architecture parameters selected for analyses were BD: Burrow diameter (mm), TBL: Total burrow length (mm) and TBD: total burrow depth (mm).

## RESULTS

There was a definite trend in the distribution area (biotope) of thèse three species of fiddler crabs. *U. chlorophthalmus* was the most frequently found species, mostly distributed from low tide level to mid tide level and to some extent up to high tide level of sandy and muddy areas. *U. sindensis* was mostly found at the high tide level of muddy and sandy areas. *U. annulipes* was mostly associated with fringing mangroves among pneumatophores.

TABLE 1

Summary of descriptive statistics for sediment properties (percentage organic, mean and median), burrows density and crab density from Sandspit backwater mangrove area and Korangi creek mangrove area during the study period.

| Crab Species (Site) | Percentage organic | Mean (Φ) | Median (Φ) | Crab Density ($m^{-2}$) | Burrow density ($m^{-2}$) |
|---|---|---|---|---|---|
| *Uca annulipes* (Sandspit S1) | 2.15±0.69 (1.28-3.56) | 1.01±0.07 (0.88 -1.13) | 0.95±0.06 (0.81-1.01) | 28±12 (4-64) | 74.5±31.0 (48 -144) |
| *Uca chlorophthalmus* (Sandspit S2) | 1.85±0.67 (1.20–3.05) | 1.29±0.16 (1.26-1.55) | 1.35±0.06 (1.25-1.42) | 45±16 (4-154) | 145.1± 80.5 (56-252) |
| *Uca sindensis* (Korangi creek) | 3.97±0.82 (2.60-4.29) | 2.31±0.11 (2.14-2.47) | 2.26±0.08 (2.13-2.36) | 13±09 (2- 44) | 98.7±52.5 (52 - 128) |

**Relationship between density ($m^{-2}$) of crabs and crabs burrows**

The density of crabs and crab burrows varied considerably for all three species. The numbers of burrows were usually greater than the number of crabs. T- tests showed significant differences between the burrow and crab densities (Df = 11, T = 7.81, P < 0.001), (Df = 10, T = 4.78, P < 0.001) (Df = 11, T = 6.28, P < 0.001) for *U. sindensis, U. annulipes* and *U. chlorophthalmus*, respectively.

**Relationship between carapace length of crabs and their burrow diameter**

The relationship between carapace length of each resident crab (CL) and its burrow diameter (BD) was identified for each studied species by using simple linear regression. The data of male and female crabs were pooled as no significant intersexual differences were observed during preliminary analyses. Good correspondence and significant positive linear relationships were observed for all three species. Linear regressions for the different species of fiddler crab are:

*U. sindensis*: CL = 2.212 + 0.591 *BD $r^2$= 0.713
*U. chlorophthalmus*: CL = 2.194 + 0.577 *BD $r^2$ = 0.727
*U. annulipes*: CL = 5.118 + 0.419 *BD $r^2$ = 0.586

**Sediment properties**

The percentage of organic matter in all the biotopes of the three investigated species varied significantly. It was highest (3.92 + 0.87) for the *U. sindensis* biotope compared to biotopes of the other two species (Table 1). Grain size showed variation in sediment composition between biotopes of the three species (Fig. 3). The mean grain size (Mz) 2.31 Φ ±0.13 Φ can be classified as fine sand for *U. sindensis,* medium sand with Mz of sediments 1.35 Φ ±0.16 Φ for the site of *U. chlorophthalmus* and as coarse to medium sand ranging between 0.99 Φ ± 0.04 Φ phi for *U. annulipes* (Table 1).

**Architecture of crabs burrows**

A total of 148 casts were made for the three species of fiddler crabs during the study period: 58 for *U. chlorophthalmus*, 45 for *U. sindensis* and 45 for *U. annulipes*. Burrow casts generally varied in length and in the shape of closed ends (forming C,

L, J, U, V and Y shapes) in vertical and complex branching morphologies with a single entrance, to complex interconnected burrow shafts with multiple entrances (Figs 4 & 5).

The burrow cast variables (TCL, TBD, BD and BV) varied according to species, their distribution according to tide levels, and biotope characteristics. Comparison of the size of burrow casts between the three species of *Uca* showed the mean TBL was 105.4±45.9 mm for *U. annulipes,* 128.0±34.2 mm for *U. chlorophthalmus* and 220.2±71.0 mm for *U. sindensis* (Table 2). The mean BD was 13.7±3.0 mm (*U. annulipes*),

TABLE 2

Summary of descriptive statistics for burrow morphological characters of three species *U. annulipes*, *U. chlorophthalmus* and *U. sindensis* from the two study sites.

| Variable | Species | N | Mean + St.Dev | Min | Max |
|---|---|---|---|---|---|
| Total burrow length (TBL) in mm | *U. annulipes* | 40 | 105.4 ± 45.9 | 45 | 210 |
| | *U. chlorophthalmus* | 58 | 128.0 ± 34.2 | 62 | 205 |
| | *U. sindensis* | 40 | 220.2 ± 71.0 | 110 | 335 |
| Total burrow depths (TBD) in mm | *U. annulipes* | 40 | 92.7 ± 36.5 | 32 | 145 |
| | *U. chlorophthalmus* | 58 | 95.7 ± 29.3 | 46 | 165 |
| | *U. sindensis* | 40 | 150.4 ± 62.0 | 70 | 244 |
| Burrow diameter (BD) in mm | *U. annulipes* | 40 | 13.7 ± 3.0 | 7 | 18 |
| | *U. chlorophthalmus* | 58 | 10.5 ± 3.8 | 5 | 26 |
| | *U. sindensis* | 40 | 12.8 ± 1.9 | 10 | 17 |
| Burrow volume (BV) $cm^3$ | *U. annulipes* | 40 | 8.47 ± 5.87 | 1.84 | 23.04 |
| | *U. chlorophthalmus* | 58 | 6.69 ± 4.11 | 1.92 | 22.47 |
| | *U. sindensis* | 40 | 34.37 ± 16.94 | 16.61 | 82.39 |

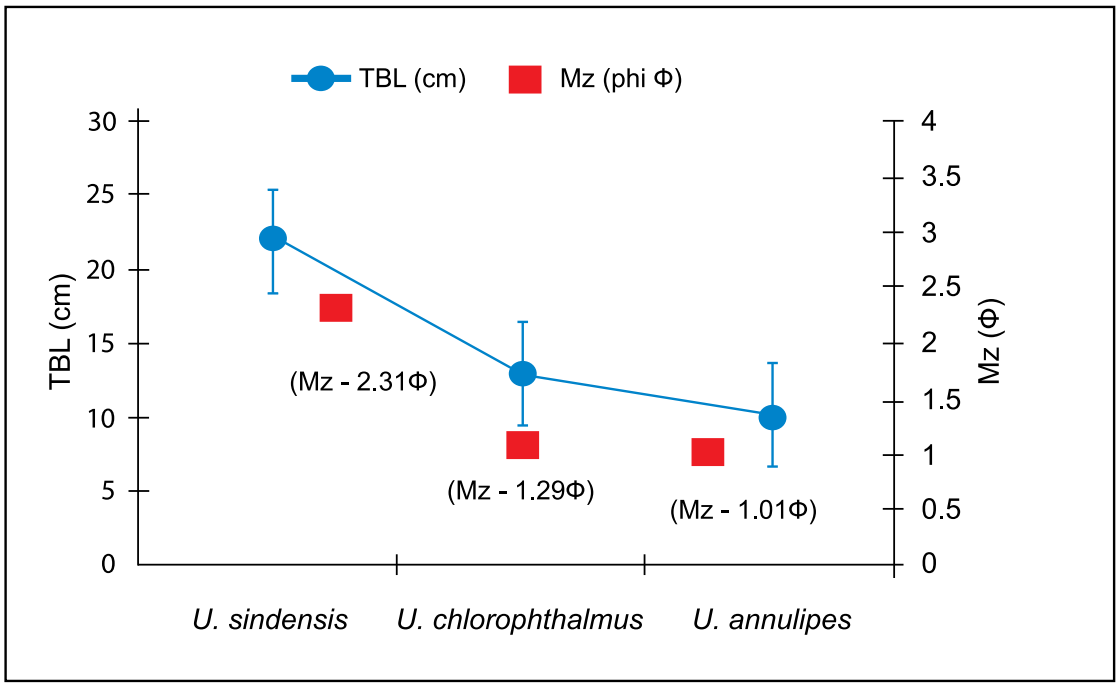


Fig. 3. – The average total burrow length (TBL) of three species of fiddler crab (*Uca sindensis, U. chlorophthalmus, U. annulipes*) in relation to mean sediment (Φ) grain size.

10.5±3.8 mm (*U. chlorophthalmus*) and 12.8±1.9 mm (*U. sindensis*) respectively. One-way ANOVA showed significant differences in TBL, TBD, BD and BV between the three species studied (Table 3). Also the density of burrow openings varied significantly ($F_{2, 17} = 24.79$, $P < 0.005$) between the three species (Table 3). *Post hoc* analysis showed that the density of burrow openings was significantly highest in *U. chlorophthalmus,* compared to *U. annulipes*, which was in turn significantly greater than *U. sindensis* (*U. chl.* > *U.ann.* > *U.sin*.) (Fig. 3).


Fig. 4. – The burrow structures of *Uca annulipes* (A) from Sandspit backwater mangrove area and *U. sindensis* (B) from Korangi creek mangrove areas during the study period.

Total burrow length, burrow depth and burrow volume were significantly greater in *U. sindensis* compared to the other two species (*U. sin.* < *U. ann.* ≈ *U. chl.*). The confidence interval for the TBL difference between the means of *U. sindensis* and *U. chlorophthalmus* extends from (4.122 to 11.654) to (-11.792 to -5.413) between the *U. annulipes* and *U. sindensis*. These ranges do not include zero, indicating that there was a statistically significant difference between the corresponding means.

The burrows of *U. annulipes* were less in volume with wider burrow openings. Burrow volume of *U. sindensis* was highest, with more winding shafts and usually only a single entrance observed (Fig. 4), whereas *U. chlorophthalmus* had multiple openings for a single burrow and variation in cast structure (Fig. 5).

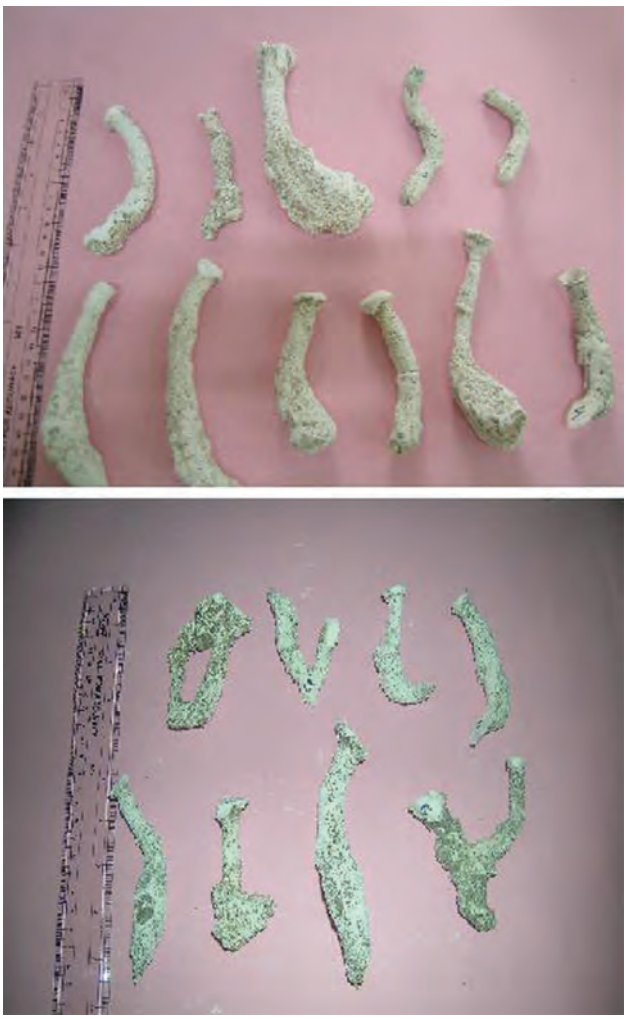

Fig. 5. – The burrow structures of *Uca chlorophthalmus* from Sandspit backwater mangrove area during the study period.

TABLE 3

Result of one way analysis of variance (ANOVA) comparing different burrow morphological characters of three species *U. annulipes*, *U. chlorophthalmus* and *U. sindensis*. (DF is degree of freedom, F is the F-statistics, and P is the probability level).

| Variable | df1, df2 | F | P | Significance | Tuckey's test |
|---|---|---|---|---|---|
| Total Number of burrows in $m^2$ | 2, 17 | 24.71 | 0.004 | * | *U. ch.< U. an.< U. si.* |
| Total Burrow Length (TBL) in mm | 2, 147 | 22.19 | 0 | * | *U. si. < U. ch.≈U. an.* |
| Total Burrow Depth (TBD) in mm | 2, 147 | 8.19 | 0.001 | * | *U. si. < U. ch.≈U. an.* |
| Burrow Diameter (BD) in mm | 2, 147 | 20.58 | 0 | * | *U. si. < U. ch.≈ U. an.* |
| Burrow Volume (BV) in $cm^{-3}$ | 2, 147 | 24.84 | 0 | * | *U. si. <U. ch. < U. an.* |

## DISCUSSION

### Relationship between density of crabs and crab burrows

It is notoriously difficult to estimate the densities of fiddler crabs in relation to burrows. Burrow densities are related to surface activities, which are related to biotic functions such as feeding, availability of food, reproductive activities, agonistic behaviour, predation and recruitment. They are also related to abiotic features such as substratum preference, harsh conditions (rise and fall in temperature), tidal periodicity etc., and can result in spatial and temporal variability and over-estimates of crab densities (Skov & Hartnoll, 2001). The use of burrow densities as surrogate to crab densities has been established in the literature (Aspey, 1978; Macintosh, 1984; Genoni, 1991; Mouton & Felder, 1995; Dray & Paula, 1998, Nobbs & McGuinness, 1999). In the present study we observed higher densities of burrows than crabs in all three investigated species. We found a significant difference in burrow-opening density between the three species of *Uca* with the highest density in *U. chlorophthalmus* correlated with the lowest amount of organic matter. Genoni (1991) tested the effect of food availability through experimental studies, and found that fiddler crabs dig more burrows despite the presence of pre-existing unoccupied burrows. As fiddler crabs mostly feed around their burrow in a circular path on open mudflats (Saher & Qureshi, in press), it is likely that additional burrows may increase feeding opportunities during periods of limited food availability and during the limited duration of low tide.

Burrows also serve other purposes such as providing escape routes from predators or fulfilling reproductive needs (Warren, 1990; Christy et al., 2001, 2002; Shih et al., 2005). Crabs construct and maintain burrows that also provide refuge from both terrestrial predators during low tide and exposed periods when it is necessary to make a hasty retreat when pursued or threatened, and from aquatic predators when flooded (Montague, 1980; Morrisey et al., 1999). In *U. chlorophthalmus*, which had burrows with more than one opening, the density of burrow openings did not give an accurate estimate of the burrows present under the ground. It is likely that burrow-opening counts are not an accurate

indicator of burrows and crab abundance. In the present study *U. chlorophthalmus* crabs dug more burrows and burrow branches of lesser depth, probably to more quickly bring nutrient and rich organic sediments to the surface, as more organic matter is present in the sediments near the surface due to the fibrous mangrove root network. The multiple entrances may allow for easy escape from predators and also provide better nutrient exchange between water and the burrow environment during high tide, and better trapping of oxygen from air during the low tide period (MORISSEY et al., 1999).

**Relationship between carapace length of crabs and burrow diameter**

Larger-sized crabs had greater burrow diameter, larger burrow volume, and bigger chamber diameter than small- and medium-sized crabs. LIM (2006) compared sympatric populations of *U. annulipes* and *U. vocans* and found that burrow architecture was similar except for wider burrow diameters of *U. vocans* and related this to significantly large carapace length to carapace width ratios of *U. vocans*. She suggested that the difference in shape of the crab carapace required wider burrow diameter in *U. vocans* to enable the crab to move comfortably into the burrow.

**Architecture of crabs burrows**

The results of our one-way ANOVA showed significant differences in all burrow cast morphological characters between the three species *U. annulipes, U. chlorophthalmus* and *U. sindensis*. The morphology of burrows is mostly species-specific (GRIFFIS & SUCHANEK, 1991; WOLFRATH, 1992). However, given the wide variety of physical and chemical differences between different sediment types and vegetation, burrowing species might modify burrow architecture to adjust to a specific set of environmental parameters (GRIFFIS & CHAVEZ, 1988).

Literature on many *Uca* species is available; the general shape of the burrows has been described as either L or J-shaped (KATZ, 1980: *U. pugnax*; GENONI, 1991: *U. rapax*; MONTAGUE, 1980: *U. pugilator*) or even U-shaped (MONTAGUE, 1980: *U. pugnax*). MONTAGUE (1980) studied burrow morphology and observed that generally all *Uca* burrows were nearly vertical and straight, mostly unbranched with an enlargement at the terminal end. The morphology of the burrows of the three species of *Uca* examined in the present study showed considerable variation in shape, size, depth and complexity between species and ranged from single entrance shafts with no branches to interconnected shafts with multiple entrances.

Comparison of the size characteristics of burrow casts showed that the mean TBL and TBD were smallest for *U. annulipes,* and largest for *U. sindensis*. The corresponding burrow diameters were smallest for *U. chlorophthalmus* and largest for *U. sindensis*. LIM & DIONG (2003) found large-sized crabs made burrows with large diameters, volumes and chamber diameters: Crabs thus resided in spacious burrows, and significantly deeper burrows were found at high shore level than at mid and low shore regions. Deeper burrows might also be a way of maintaining lower burrow temperatures in areas that are exposed to sunlight for greater periods of time (POWERS & COLE, 1976; WOLFRATH, 1992; LIM & DIONG, 2003). We found the deepest burrows for *U. sindensis*, where the burrow area is covered during high spring tides and consists of open mudflats devoid of any vegetation, whereas the burrows of *U. annulipes* were found at shallow and intermediate depths and are regularly flooded by daily tides. This pattern found in the present study is consistent with that found for other crabs with deeper burrows in drier sediments (TAKEDA & KURIHARA, 1987; WOLFRATH, 1992; LIM & DIONG, 2003). THURMAN, (1984) showed that burrows of *U. subcylindrica* increase in depth with increasing distance from low water mark in Laguna Salado, Mexico, while WOLFRATH (1992) reported that burrows of *U. tangeri* were deeper at high tide levels of salt marsh than at lower levels near the water front. Burrows do not necessarily contain standing water but are usually only damp at low tides, and fiddler crabs have been observed to

return to them every 10 to 30 minutes during feeding to renew the respiratory water lost both through evapo-transpiration and feeding activities (Power, 1975; Montague, 1980; Saher & Qureshi, in press).

Lim & Diong (2003) further hypothesized that deeper burrows in the high intertidal areas might help fiddler crabs to maintain lower burrow temperature during ebb tides. They also observed that burrows in anoxic sediments had significantly shorter depths, which would help to improve aeration. Thus, a combination of temperature and moisture levels in the substratum could influence the burrow depth architecture for *Uca* species, playing an important ecological role in the life history of these species.

The surrounding vegetation and general sediment characteristics were found to be important factors influencing the burrow morphology of these species. Previous studies on ocypodid crabs found that crabs preferentially dug burrows near hard structures, such as plants and experimental enclosures, because these structures provided the burrows with some structural support (Bertness & Miller, 1984; Lim & Rosiah, 2007). The presence of vegetation has also been found to alter the stability of sediments (Bertness & Miller, 1984). In the present study, the burrows of *U. annulipes* were distributed among pneumatophores and had thicker root matter in the burrow soil. Despite a comparative lack of vegetation, the burrows of *U. sindensis* were more winding and burrows of *U. chlorophthalmus* were wider than those of *U. annulipes*, which mostly consisted of straight shafts. In addition, many crabs can vary their burrowing behavior in response to the characteristics of the sediment (Genoni, 1991; Ewa-Oboho, 1993; Klassen & Ens, 1993). The sediments at sites of *U. annulipes* were coarser than at sites where we found *U. chlorophthalmus* and *U. sindensis*, and coarser sediments might have restricted deeper burrowing to some extent in *U. annulipes* as burrowing in coarser sediments is energetically more costly (Grow, 1982). The comparative lack of vegetation and fine sediments enabled *U. sindensis* to construct wide and deep burrows, winding below the surface to increase the space available inside the burrow within a limited depth (Fig. 4). The larger burrow volume and increased surface area would allow for a larger volume of air to aerate the water that collects in the burrow when submerged during high tide (Lim & Diong, 2003). Finer sediments might provide a more stable substrate for larger, more complex burrows owing to their more cohesive nature (Takeda & Kurihara, 1987; Rudnick et al., 2005). Therefore, the presence of vegetation and the sediment properties in the present study could account for the differences observed in the patterns of burrow structure between all three species.

## CONCLUSIONS

In conclusion, our results show that *Uca* species display significant interspecific variation in their burrow morphology in relation to the biotic and abiotic factors of their biotopes, such as sediment composition, substratum hardness and root-mat density of the surrounding vegetation. The sediment characteristics (percent organics, composition) and vegetation cover influence the morphology of fiddler crab burrows. The various combinations of sediment type and presence of vegetation as well as other environmental variables, such as inundation levels, result in burrows of different structure. More work is required to determine the extent of the impact of these environmental factors on burrow morphology. However, the spatial variation in burrow morphology found in the present study suggests that the varying burrowing activity of the investigated crabs in relation to vegetation and sediment composition is likely to be species specific and this should be further addressed in future studies.

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# SHORT NOTES

## Larval habitat characteristics along the Scheldt estuarium of *Bradysia ocellaris* (COMSTOCK), a Black Fungus Gnat (Diptera: Sciaridae) of economic importance

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KEY WORDS: Diptera, Sciaridae, Belgium, Scheldt estuarium, emergence traps.

Species of the family Sciaridae (Diptera: Sciaroidea) are found worldwide and more than 1,700 species have been described so far (7). From Europe 649 species have been identified to date (5) of which 35 are recorded in Belgium (6). These flies have largely been neglected because of their small size (1–7 mm), their hidden lifestyle and the difficulties involved with their identification. Sciarid larvae live in decaying organic matter in the soil, under bark of decaying trees and on fungal fruiting bodies (1). The larvae of some species, however, can also be found in leaves and stems of plants (3). Some species of Sciaridae are common pests in greenhouses (2).

In spring 2009 large numbers of larvae of *Bradysia ocellaris* (COMSTOCK, 1882) were found in mud samples (first 5 cm) from the tidal zone along the river Scheldt near Gentbrugge. This species had not been previously recorded in Belgium (4). Probably the larvae feed on fungi and rotting plant material that are present in the tidal mud.

In Europe, *B. ocellaris* can be a common and serious economic pest of glasshouse crops, and is sometimes found in association with the cultivation of the mushroom *Agaricus bisporus* LANGE (IMBACH) (Agaricaceae) (8). Mass occurrences of larvae are not uncommon and can cause extensive damage in forestry and agriculture (8), leading to significant losses in crop production (7). In the neighbourhood of Gentbrugge near the river Scheldt no large greenhouses can be found, but several companies have storage rooms for food articles, and also private gardens with small greenhouses are present in this area. In order to understand how and when *B. ocellaris* specimens could become a pest in areas surrounding the river Scheldt, experiments were carried out to determine some ecological characteristics and larval survival of this species under changing water levels in the tidal zone of the river.

On 15 March 2009 15 mud samples (1000 ml) were taken into the laboratory. Each sample was divided into five subsamples (200 ml). Four of these subsamples were given different treatments for 46 days (subsample A: 6 days submerged under 10 cm water from the Scheldt and afterwards kept humid; subsample B: let dry out; subsample C: kept humid with water from the Scheldt and subsample D: 15 days submerged under 10 cm water from the Scheldt and afterwards kept humid. All the subsamples

with different treatments were kept in emergence traps (14 cm high and diameter of 9 cm). All traps were exposed to the same temperature (26 °C), light regime (8 h dark and 16 h light) and humidity. The fifth subsample was used to determine relative moisture and relative organic matter content.

Subsamples for analysis of organic matter were dried at 105 °C for 24 h. Afterwards they were weighed, placed in the oven at 430 °C for 24 h and reweighed. The difference between the weights corresponded to the total organic matter, which was expressed as a percentage. Subsamples for analysis of relative moisture were weighed to determine their moist weight, dried and then reweighed to determine the soil moisture content. The weight difference was expressed as a percentage of the original weight.

The preparation and identification of all emerged male sciarid specimens was conducted by Frank Menzel. Sciarid specimens were prepared as permanent mounts for microscopic examination using Canada balsam as mountant. They were deposited in the collections of Senckenberg Deutsches Entomologisches Institut, Müncheberg, Germany (76 ♂♂) and Royal Belgian Institute of Natural Sciences, Brussels, Belgium (37 ♂♂). Identification of female sciarid specimens to species level is not possible based on morphological characteristics alone. However as all males belonged to one species we assumed all females to be *B. ocellaris* too. The first *B. ocellaris* were observed after 11 days (26-03-2009) and 625 individuals of *B. ocellaris* emerged from the treatments in total (Table 1).

The organic matter content of the samples from which *B. ocellaris* emerged varied between 9 and 22 %. The range for the percentage of relative moisture was 45 to 62.5 %.

There was a significant difference between the different treatments ($F_{3,56} = 22.51$, $p = < 0.001$). No correlation was found between the relative moisture content of the original sample and the number of *B. ocellaris* emerging for any of the treatments, suggesting no preference for a certain humidity of the mud samples. We did find a significant positive correlation between the organic matter content of the samples and the number of adult *B. ocellaris* in treatment C (kept humid) ($p = 0{,}0423$) (Fig. 1).

A lot of moist decaying organic matter suitable as food for the larvae is present at the Sea Scheldt arm in Gentbrugge favouring the presence of high abundances of *B. ocellaris*.

*Bradysia ocellaris* is regarded as an introduced species (2) and is mainly found in greenhouses (8) where they are mostly introduced with contaminated soil (2). Here it seems, that the species has developed a temporary mass occurrence under favourable conditions in the estuary ecosystem at the Sea Scheldt arm in Gentbrugge. The migration of these flies from small greenhouses of public gardens in the vicinity of Gentbrugge to the tidal zone of the river Scheldt near Gentbrugge is a possibility. However it still remains the question if such a mass occurrence might serve as a source for nearby *B. ocellaris*-free greenhouses in the neighbourhood. We therefore suggest a detailed monitoring of potential new habitats in the neighbourhood.

TABLE 1

Number of emerged *B. ocellaris* for each treatment.

| **Treatment** | **Mean *B. ocellaris*/subsample** | **Total *B. ocellaris*/treatment** |
|---|---|---|
| A (submerged for 6 days) | 16,2 | 243 |
| B (let dry out) | 1,1 | 17 |
| C (kept humid) | 24,3 | 365 |
| D (submerged for 15 days) | 0 | 0 |
| Total | | 625 |

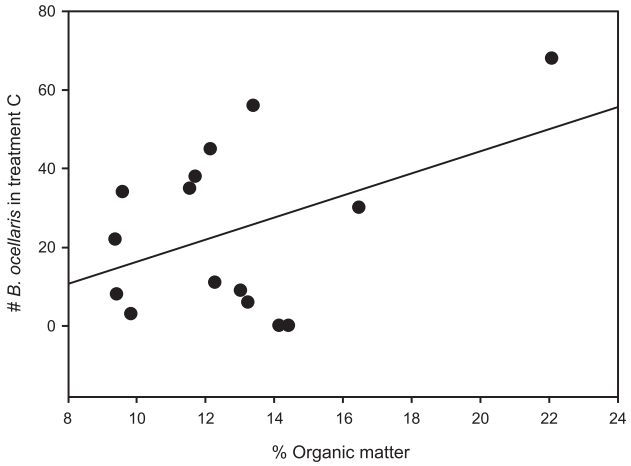


Fig. 1. − Organic matter (%) in relation to the number of *B. ocellaris* (ordinate) in treatment C (samples that were kept humid)

Sciaridae are terrestrial organisms, which prefer semi humid conditions, so the presence of *B. ocellaris* in a tidal habitat is remarkable. In our experiments, we found that when the larval habitat was submerged for 15 days, no larvae survived. Submerging the larval habitat for 15 days could, therefore, be an efficient way to prevent the emergence of adult *B. ocellaris* and their slater colonisation of urban regions near the Scheldt. Submerging the area for even a shorter period could reduce the number of adult *B. ocellaris*. We therefore suggest such a management action if problems with Black Fungus gnats appear in the urban surrounds of the Scheldt.

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# The effect of insertion of a dummy acoustic transmitter on the survival of pouting, *Trisopterus luscus* L.

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Acoustic fish telemetry is an often used technology that can provide valuable data on fish movement, behaviour and habitat use. In recent years, many novel applications and ameliorated transmitter designs have made it an increasingly popular tool in fisheries research (1-4), resulting in substantially improved knowledge on behavioural, ecological and physical issues (5-9) of many fish species in previously out-of-reach environments. HEUPEL et al. (1) stated "any aquatic species to which a transmitter can be attached or implanted without modifying the behaviour of the animal is potentially suited to this technology". The size of the transmitter and the disturbance to a fish should be minimized in order to study the fish behaviour (10).

Monitoring of fish communities in wind farms in the Belgian part of the North Sea (BPNS) revealed that pouting, *Trisopterus luscus* (LINNAEUS, 1758), was present in high densities in the vicinity of the wind turbines during parts of the year. There is evidence that food availability for pouting increased at these wind turbines (11). To study the spatio-temporal migration and site fidelity of pouting at the offshore wind farms, we plan to use acoustic telemetry. However, pouting is a very sensitive species which survives manipulations only in very low percentages (pers. observations). As knowledge of survival rates is indispensable to an assessment of the likelihood of success of a tagging experiment, a laboratory experiment was set up to investigate the potential for pouting to be used in acoustic telemetry studies. To our knowledge, this is the first experimental study in acoustic telemetry on pouting and the information obtained could be valuable for future applications.

The pouting individuals used in the experiment were collected at a wind farm in the BPNS, using hook and line gear. After capture, fish were kept in an aerated water tank for transportation to aquarium facilities (water temperature of 14°C) at the Institute for Agricultural and Fisheries Research. After an acclimatisation period of five to seven days, the fish were starved for two days (12) before the surgical operation, in order to maximize the intestinal space for tag insertion. Surgical procedures were similar to those of BARAS & JEANDRAIN (13), ARENDT et al. (14) and JADOT et al. (15). Prior to tagging, the fish were anaesthetized in a 0.3ml $l^{-1}$ 2-phenoxyethanol solution. Following anaesthesia, the fish, showing no reaction to external stimuli, slow opercular rate and loss of equilibrium (16), were placed ventral side up in a V-shaped support. Most of the body, except the ventral side, stayed in the water and a continuous flow of aerated water was pumped over the gills to avoid gill damage and to provide a continuous oxygen supply (17). A small incision (15-22 mm) was made on the mid-ventral line and a dummy acoustic transmitter (Vemco, coded, V9-1L) was inserted in the visceral cavity. The incision was closed with two sutures (polyamide

monofilament, DS19 3/0). All instruments and transmitters used were disinfected with iso-betadine®. In total 15 specimens were tagged with a dummy transmitter. The 10 specimens of the control group were anaesthetised to mimic the handling procedure.

After the surgical procedures all pouting were stocked together in a fish tank (2 x 2 x 0.5m³) on recirculation (i.e. a closed system in which no extra water is added). The tank was checked daily for survival and tag retention. Pouting were fed with fish fillets. The experiment ran for six weeks.

Fish survival rates were compared using chi-square tests. A Two-way contingency table was constructed for survival (dead-alive)/treatment (tag-control) comparison. Statistical analysis was performed in R (version 2.5.1 www.r-project.org). T-tests on the difference in total length of pouting between the treatments were carried out in Statistica (version 7.0, Statsoft, Tulsa, Oklahoma). A significance level of $p < 0.05$ was used in the tests and results expressed as mean ± SD.

The fish length varied between 14.5 cm and 27.5 cm, and between 17.3 cm and 28.5 cm for the tagged and control group respectively. No significant differences in length were present between the groups (T-test, $p = 0.49$). In the first week after surgery a significant difference in survival rate ($\chi^2$-test, $p = 0.041$) was detected between the tagged group (survival: 66.7 %) and the control group (survival: 100 %). Data screening showed that there was a tendency to better survival in larger fish within the tagged group. The fish that died had an average length of 20.5 ± 3.5 cm, while the fish that survived had an average length of 23.2 ± 4.2 cm. However, no significant differences in length were present between the groups (T-test, $p = 0.24$). From the second week onwards there was no further mortality in either group. However, one tagged fish expelled its dummy transmitter in the third week. During the whole period of the experiment all fish ate well and a small increase in length was observed. In the tagged group, overall average length increased from 22.8 ± 4.3 cm to 23.2 ± 4.2 cm, while in the control group it increased from 23.0 ± 4.2 cm to 23.2 ± 3.9 cm. Individual length increment was not monitored as several individuals lost their external identification tag during the experiment. Only fish that survived the experiment were used to calculate average lengths. The experiment took place in the run-up to the spawning season and post-mortem investigation revealed that some specimens had maturing gonads.

Although the experiment was small-scale (due to the limited number of pouting that could be caught), some clear trends were revealed. Tagged individuals had a significantly lower survival probability compared with non-tagged individuals. The results suggest that survival may be influenced by length. Larger animals tended to have higher survival chances, compared with smaller specimens. The experiment clearly showed that if tagged animals died, it was within the first week after surgical procedures. Therefore, we suggest that pouting does have the potential to be used in telemetry experiments.

However, as survival is indispensable to the maximum likelihood of success of a tagging experiment, only animals in good condition should be released. Therefore, fish should be monitored for an observation period of one week after surgical procedures to allow them to recover from stressors (18). In addition, specimens above a minimum length should be used. We suggest this minimum to be at least 23 cm, which is the average length of the tagged fish that survived. All pouting in captivity ate well, increased slightly in length and their gonads matured, so it appeared that tagging did not influence their growth and feeding behaviour.

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# The Pied and the Collared Flycatcher do not compete for microhabitats in the Białowieża Forest

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When resources are limited two species often compete, which leads to negative fitness consequences as for example with the Great Tit *Parus major* and Blue Tit *Cyanistes caeruleus* (1) or Great Tit and Pied Flycatcher (2). Our study investigates the opposite situation - what happens with two sibling species when resources are not limited but under primeval conditions? Pied Flycatcher *Ficedula hypoleuca* (PALLAS, 1764) and Collared Flycatcher *F. albicollis* (TEMMINCK, 1835) are closely related species, with similar breeding ecology regarding nest sites, food type and foraging techniques. In areas of overlapping distributions, the Collared Flycatcher (CF) is often numerous, while the Pied Flycatcher (PF) breeds in much lower densities (e.g. 3; 4; 5). Competition between these species has been described many times (e.g. 3; 6; 7). In managed forests, both species compete for nest boxes and, as a result, PFs are forced to breed mostly in poorer, coniferous stands (8). Under the primeval conditions of the Białowieża Forest (NE Poland) CF is very numerous only in deciduous stands, while PF breeds in very low densities in all habitats (9; 10; 11; 12). Thus, both species coexist in deciduous stands where no nest boxes are present, and they breed only in tree holes. It is not known, however, whether PF and CF differ in microhabitat use within primeval deciduous stands, where resources are not limited.

The goal of this work was to examine how PF and CF differ in microhabitat use in deciduous stands under primeval conditions. To achieve this goal, we compared microhabitat characteristics surrounding the nest cavities of both species in two deciduous forest types within the primeval stands of the Białowieża Forest.

The study was conducted in the Białowieża National Park (hereafter abbreviated as BNP; 52°41'N, 23°52'E). The strictly protected part (47.5 km$^2$) has never been logged or planted. The structure of the forest there is of uneven age with multi-layers and multiple tree species. Lime-hornbeam-oak *Tilio-Carpinetum* stands are the dominant type in this forest being mainly composed of lime *Tilia cordata*, hornbeam *Carpinus betulus*, Norway spruce *Picea abies*, oak *Quercus robur*, maple *Acer platanoides*, and elms *Ulmus* spp. The dominant species in ash-alder *Circaeo-Alnetum* stands are alder *Alnus glutinosa*, ash *Fraxinus excelsior* and Norway spruce.

Data were collected in 1997 – 1999 within permanent ornithological study plots, in lime-hornbeam-oak stands (three study plots, in total 138 ha) and in an ash-alder riverine stand (one plot, 33 ha; 9). We searched for PF and CF nest cavities (methods described in WALANKIEWICZ et al. (13)). Due to the low density of PF, searches for their tree cavities were also conducted outside the study plots, in stands adjacent to the plots, and along the roads of the BNP. In July and August, we measured the habitat characteristics around

nest cavity trees and in random plots, which were chosen in the vicinity of the flycatcher sample plots. We assumed that a circle with a 20 m radius (0.126 ha) around the nest cavity tree was sufficiently large to characterize the birds' nesting habitat use. We measured within each sample plot the tree crown cover (the vertical projection of crown cover was drawn on a map, from which a total share of canopy cover was calculated as percentage), the composition of tree species, the condition of all trees (living or standing dead as snag) and the diameter of the tree trunks at breast height (DBH).

All trees with a DBH ≥ 12 cm were included in the category "trees", thinner ones into the category "saplings". For each measured tree, the basal area was calculated based on its DBH (as the round area of the tree trunk section at the breast height). Then, basal areas of all trees within each plot were added, and this sum was used for analysis as a basal area of the flycatcher/random plots. In lime-hornbeam-oak stands, 34 PF plots, 36 CF plots, and 52 random plots were measured. In ash-alder stands 10 PF, 14 CF, and 21 random plots were measured.

Generalized linear mixed models (GLMM), computed with the freely accessible statistical software R (R 2.10.0), were used to analyze microhabitat selection. With this multiple logistic regression we quantified the relationship between several predictor variables (habitat properties) and a response variable (presence and absence data). This is a standard approach in habitat selection analysis (14). We computed three types of GLMMs: the first was composed of plots with PF and plots with CF, the second was composed of plots with PF and random non-occupied plots, while the third was composed of plots with CF and random non-occupied plots. The habitat selection analyses with full GLMM models involved seven variables: 1) crown cover, 2) basal area of living trees, 3) basal area of snags, 4) number of living trees, 5) number of snags, 6) number of saplings and 7) number of spruces. The habitat type (lime-hornbeam-oak stand (L-H) or ash-alder stand (A-A)) was included as a random factor.

We identified the best model by stepwise omission of non-significant terms. The inflection point of the fitted logistic regression function, where the estimated probability of species presence equals 0.5, was used to classify habitat according to suitability for each species. The classification performance was tested with a chi-square test of independence (adjusted with Yates' correction for continuity). This was computed on the "confusion matrix" composed of frequencies of correctly and wrongly assigned presences or absences. The percentage data were transformed using arcsine square root transformation prior to analysis into data that were close to a normal distribution (15). For comparison of tree stand composition between sample plots, we used a G-test.

In the full model comparing the habitat used by the two flycatcher species, none of the terms were statistically significant predictors. In the reduced model that contained a single predictor, basal area of living trees, the predictor was almost significant ($z=-1.95$, $p=0.051$) and the intercept was significant ($z=2.04$, $p=0.041$). The model indicates that basal area of living trees is slightly larger in PF sample plots than in CF sample plots (Table 1). The full model correctly classified 34.1% of plots used by PF and 78.0% of CF plots ($\chi^2=16.43$, df=1, $p<0.001$). The reduced model correctly classified 45.5% of plots used by PF and 72.0% of plots used by CF ($\chi^2=2.38$, df=1, $p=0.123$).

In the second model, we compared random unoccupied sample plots with those used by each flycatcher species. In the full model estimating the presence probability of PF, crown cover ($z=-2.43$, $p=0.015$), number of saplings ($z=2.04$, $p=0.041$) and number of spruces ($z=-2.12$, $p=0.034$) were statistically significant predictors. The full model correctly classified 31.8% of plots used by PF and 83.3% of unoccupied plots ($\chi^2=4.47$, df=1, $p=0.034$). In the reduced model, PF sample plots differed from random ones in all four covariates: crown cover ($z=-2.54$, $p=0.011$), basal area of living trees ($z=2.23$, $p=0.026$), number of saplings ($z=2.14$, $p=0.032$), and number of spruces ($z=-2.11$, $p=0.035$), and the

TABLE 1

The average values of sample plot parameters (per plot 0.126 ha, with ± standard deviation, and sample size in parentheses) for two flycatcher species and random plots in the Białowieża National Park. L-H – lime-hornbeam-oak stand, A-A – ash-alder stand.

| | Habitat | *F. hypoleuca* (Pallas, 1764) | *F. albicollis* (Temminck, 1835) | Random plots |
|---|---|---|---|---|
| Crown cover (%) | L-H | 88.0 ± 6.8 (34) | 87.8 ± 9.3 (36) | 92.1 ± 5.9 (52) |
| | A-A | 87.5 ± 6.8 (10) | 86.5 ± 5.8 (14) | 83.9 ± 9.8 (21) |
| No. of live trees | L-H | 35.1 ± 7.3 (34) | 31.1 ± 5.8 (36) | 35.1 ± 8.1 (52) |
| | A-A | 36.0 ± 9.3 (10) | 38.3 ± 14.4 (14) | 33.6 ± 8.6 (21) |
| Live trees basal area ($m^2$) | L-H | 4.2 ± 0.9 (34) | 3.7 ± 1.0 (36) | 3.9 ± 1.0 (52) |
| | A-A | 4.9 ± 1.4 (10) | 4.5 ± 1.1 (14) | 4.2 ± 1.4 (21) |
| No. of snags | L-H | 2.3 ± 1.8 (34) | 2.3 ± 1.9 (36) | 2.1 ± 2.2 (52) |
| | A-A | 2.1 ± 0.6 (10) | 1.9 ± 1.2 (14) | 2.6 ± 2.0 (21) |
| Snags basal area ($m^2$) | L-H | 0.3 ± 0.4 (34) | 0.4 ± 0.4 (36) | 0.3 ± 0.4 (52) |
| | A-A | 0.5 ± 0.3 (10) | 0.2 ± 0.2 (14) | 0.3 ± 0.4 (21) |
| No. of saplings | L-H | 33.1 ± 21.6 (34) | 33.2 ± 19.2 (36) | 32.0 ± 17.0 (52) |
| | A-A | 0.5 ± 0.3 (10) | 0.2 ± 0.2 (14) | 0.3 ± 0.4 (21) |
| No. of spruces | L-H | 4.7 ± 3.8 (34) | 5.6 ± 3.6 (36) | 5.0 ± 3.7 (52) |
| | A-A | 5.3 ± 3.8 (10) | 3.1 ± 2.2 (14) | 7.6 ± 6.2 (21) |

intercept was not significant ($z=1.46$, $p=0.144$). The reduced model correctly classified 34.1% of plots used by PF and 87.0% of unoccupied plots ($\chi^2=6.70$, $df=1$, $p=0.080$).

None of the predictor variables in the full model for presence of CF reached statistical significance, although crown cover was close to it ($z=-1.95$, $p=0.051$). The full model correctly classified 28.0% of plots used by CF and 87.7% of unoccupied plots ($\chi^2=42.21$, $df=1$, $p<0.001$). The reduced model contained a single predictor variable, crown cover, which explained differences between sample plots with and without CF's nests with high significance ($z=3.99$, $p<0.001$, intercept: $z=1.47$, $p<0.001$). The model correctly classified 90.0% of plots used by CF, but only 15.1% of unoccupied plots ($\chi^2=0.30$, $df=1$, $p=0.300$). Tree species composition within PF and CF sample plots was similar ($G=4.53$, $df=6$, $p=0.61$ in lime-hornbeam-oak, and $G=4.17$, $df=7$, $p=0.76$ in ash-alder tree stands).

It has been experimentally shown that PF and CF prefer different microhabitats in sympatric populations (16, 17). However, most of the publications on *Ficedula* flycatchers come from studies in managed forests that have been deeply transformed, and where there is a shortage of nest sites. We found that PF and CF microhabitats were similar under the primeval Białowieża conditions. Models predicting the presence of each species showed that both PF and CF are selective in their choice of breeding habitats, because these differed from random unoccupied plots. The Collared Flycatcher's habitats could be reliably classified solely by their low crown cover, while PF habitats seem to be additionally characterized by high basal area, high number of saplings and low number of spruce trees. However, the role of these predictors was minute in comparison to the importance of low crown cover. This additionally demonstrates the similarity of the habitat preferred by both species. Correct classifications of plots used by flycatchers were on a low level, random plots were classified

as flycatcher plots or else the results of the model were not statistically significant. In other words, PF could freely choose its breeding microhabitat (at least at the level we have studied), possibly because the most important resources for breeding *Ficedula* flycatchers, namely tree cavities, are very abundant in primeval stands of the Białowieża Forest (18). Furthermore, there is other circumstantial evidence supporting our above findings, i.e. long-term studies conducted in BNP showed both *Ficedula* species fluctuated in breeding numbers in parallel (e.g. 5, 9, 10, 11). Therefore, the lower abundance of PF cannot be explained by competitive pressure from CF.

We conclude that the Collared Flycatcher did not force the Pied Flycatcher to use different microhabitats under primeval conditions but both species chose nesting spots freely. It is furthermore worth emphasising that both species showed similar microhabitat preferences under primeval conditions. Finally, we suggest that competition between PF and CF as described (e.g. 3; 8; 19) is most likely a result of forest management and nest box supply.

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# Distribution and ecology of mosquito larvae (Diptera: Culicidae) in Flanders (Belgium)

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Increasing globalization of transport of people and goods, together with climate change, create suitable conditions for the introduction of alien mosquito species as well as the (re)emergence of diseases transmitted by mosquitoes in Europe (1). Invading and alien species, as well as native species, can act as vectors of arboviruses (viruses transmitted by arthropods). Recent outbreaks of mosquito-borne diseases in Western Europe show the need for better knowledge of the taxonomic and functional biodiversity of both native and invasive vector species. To assess the distribution of adult mosquito species in Belgium a large scale inventory project was launched in 2007: the MODIRISK-project (1). This project resulted in an overview of the distribution and habitat niche of adult mosquitoes in Belgium. However, larvae were neither collected nor surveyed and there is still a gap in information on larval-habitat characteristics of Belgian mosquitoes. Especially the niche of the larvae of some common species that are known as vector for arboviruses is still poorly known in Belgium and Flanders. Here, distribution maps for the encountered species are plotted and habitat requirements are investigated by assessing the circumstances in which the different taxa are found.

In the context of water quality monitoring by the Flemish Environment Agency, macroinvertebrates have been sampled at several thousand sampling points since 1989. However, it was only in 1997 that the sampling network became really extensive and therefore, material from 1997-2009 was studied. Since water quality monitoring has mainly been focusing on running waters, stagnant waters are underrepresented. During monitoring, macroinvertebrates were

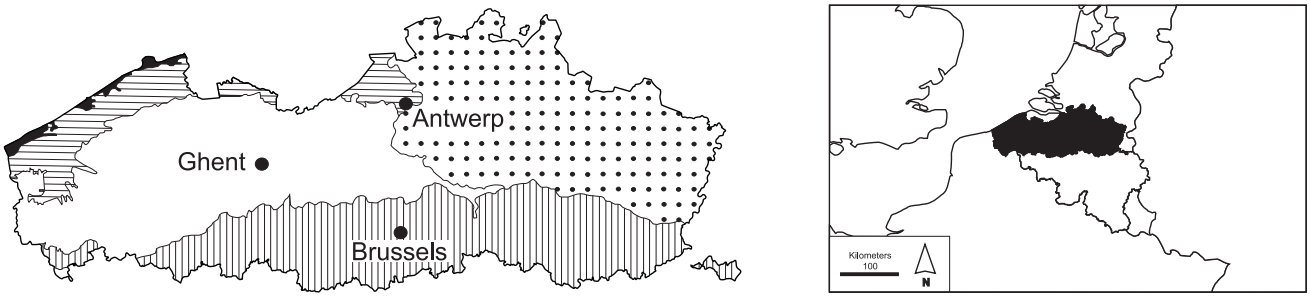


Fig. 1. – Map of Flanders with indication of the different ecoregions: dune area (black), polder area (horizontal stripes), sandy region (white), Campine region (dots) and loamy region (vertical stripes); the location of Flanders has been marked on the map.

sampled using a standard handnet, as described by GABRIELS et al. (2). At each sampling point, conductivity, pH, oxygen content and water temperature were measured. All sampled mosquito larvae were identified to the lowest possible level, which was in most cases possible up to the species, by using the identification key by SCHAFFNER et al. (3) and we used the traditional taxonomical classification (pre-REINERT, 4). In Flanders, a typology of the watercourses has been made by JOCHEMS et al. (5). The main separation of the types was based on the catchment area and in addition, the watercourses in the polder area were separated from the remaining watercourses and differentiation was made between small and large brooks from the different ecoregions: sandy region, the loamy region and Campine region. A map of Flanders with indication of the ecoregions is presented in Figure 1.

In the present study, 5823 mosquito larvae were identified, belonging to nine different taxa, however, only four species were encountered on more than three occasions. Distribution maps are given in Figure 2, while the water type where the different taxa were found from 1997-2009 is indicated in Table 1. *Anopheles claviger* (MEIGEN, 1804) mostly occurred in small streams, but avoided polder watercourses. *Anopheles maculipennis s.l.* was also sampled in larger watercourses as well as polder watercourses and stagnant waters, but was rarely found in the loamy region. *Culex pipiens* LINNAEUS 1758 is a ubiquist that was found in most water types, whereas *Culiseta annulata* (SCHRANK, 1776) occurred predominantly in small streams. *Aedes caspius* (PALLAS, 1771)*/dorsalis* (MEIGEN, 1830) was only found once in a brackish polder watercourse that was oversaturated with oxygen. *Culex territans* WALKER 1856, *Aedes punctor* (KIRBY, 1837) and *Aedes rusticus* (ROSSI, 1779) were only caught on a few occasions in small brooks, while *Culiseta morsitans* (THEOBALD, 1901) was only captured once in a large brook.

*Anopheles claviger* (median 5.6mg $O_2.L^{-1}$) and *An. maculipennis s.l.* (6.4mg $O_2.L^{-1}$) tolerated a wide range of oxygen concentrations, but *Cx. pipiens* (4.8mg $O_2.L^{-1}$) and *Cs. annulata* (2.9mg $O_2.L^{-1}$) even occurred at significantly lower oxygen concentrations (Kruskal-Wallis ANOVA followed by multiple comparisons). These four species all preferred slightly alkaline waters with a pH between 7 and 8 and tolerated a wide range

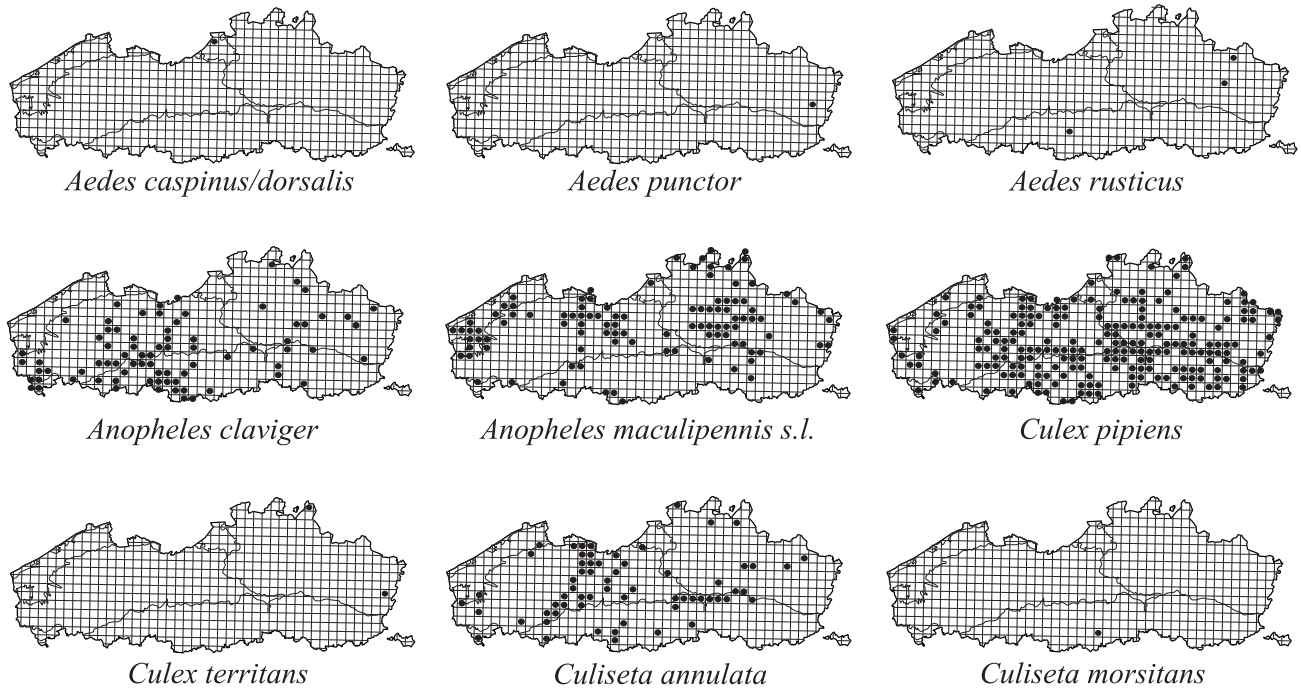


Fig. 2. – Distribution of the sampled mosquitoes in Flanders, with indication of the ecoregions and a grid of 5*5 km UTM-squares.

Table 1

Number of records of mosquito larvae per water type in Flanders from 1997 to 2009.

| Water type | Large river | Small river | Large brook | | | Small brook | | | Polder water-course | Circum-neutral lake | Alkaline lake |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ecoregion | | | Campine | Loamy | Sandy | Campine | Loamy | Sandy | Polder | | |
| Catchment area (km²) | 600-10000 | 300-600 | 50-300 | 50-300 | 50-300 | < 50 | < 50 | < 50 | Not applicable | | |
| pH | | | | | | | | | | 6.5-7.5 | >7.5 |
| *Aedes caspius/dorsalis* | | | | | | | | | 1 | | |
| *Aedes punctor* | | | | | | 1 | | | | | |
| *Aedes rusticus* | | | | | | 2 | 1 | | | | |
| *Anopheles claviger* | 1 | 1 | 3 | 3 | 2 | 14 | 32 | 41 | | | 3 |
| *Anopheles maculipennis s.l.* | 6 | 14 | 47 | 3 | 3 | 37 | 4 | 44 | 29 | 1 | 16 |
| *Culex pipiens* | 5 | 6 | 18 | 20 | 12 | 66 | 90 | 121 | 15 | | 4 |
| *Culex territans* | | | | | | 1 | | | | | 1 |
| *Culiseta annulata* | | 1 | | | 2 | 10 | 11 | 43 | 5 | 1 | 2 |
| *Culiseta morsitans* | | | | 1 | | | | | | | |

of conductivities, often surpassing 1000µS. $cm^{-1}$. However, some rarer species such as *Cx. territans*, *Ae. punctor* and *Ae. rusticus* were restricted to waters with a lower conductivity.

Only nine taxa were encountered during the present study, whereas 32 species have been reported from Belgium (6). However, only Flanders was studied and it is known that several species are restricted to Wallonia, the southern part of the country. In addition, some habitat types typical for mosquito larvae, such as temporal waters, tree holes, fens and peat boxes were not sampled, while stagnant waters were also underrepresented. Moreover, larvae of some species are often found in man-made habitats. During the present study, only surface waters (mainly running waters) were studied and data presented on the distribution and ecology of larvae will therefore inevitably be incomplete.

Some of the species encountered here are very widespread in all kinds of sampled waters and are potential vectors of arboviruses. Most interesting are *Cx. pipiens* and the two species of *Anopheles* that were found. The first, a potential vector for West Nile virus and very abundant and widespread, seems to have a wide range of potential larval breeding sites, including man-made breeding sites (7). In many regions in Flanders, West Nile virus is therefore a candidate for circulation in nature reserves but also near human settlements as it can be maintained in an avian-mosquito cycle (7). West Nile virus outbreaks are increasing in Central Europe (7) and due to global warming, these outbreaks can also be expected in Flanders in the near future. Studies on ecological delineation of the larval-breeding sites of *Anopheles* species are rare, although two taxa turned out to be widespread during the present study. *Anopheles claviger*, a primary vector of human malaria in the Middle-East, occurs in the Netherlands mostly in permanent pools, shallow canals and ditches, with a preference for shaded sites (7). Although Flanders is still too cold for human malaria, this could change if the temperature keeps rising due to global warming. Here, the larvae of *An. claviger* were found in a wide range of water types, making this species also a top potential candidate as vector for Tahyna and Batai viruses in Flanders in the future (8). The *An. maculipennis* s.l. complex contains several species that are primary vectors for human malaria and different arboviruses. In the Netherlands, three species occur: *An. messeae*, *An. atropravus* and *An. maculipennis* s.s. There, the species complex seems to occupy a wide range of water types and is widely distributed (9), which is also the case in Flanders, where it might act as a potential vector for different diseases.

Especially in some large nature reserves with high abundances of migratory birds during the mosquito season, some arboviruses can be maintained in an avian-mosquito cycle (7). In addition, there is a worldwide trend to construct, restore and protect wetlands and to improve the ecological water quality, which creates suitable habitats in which host, vectors and pathogens can come into contact. Thus at these sites, vectors might be exposed to pathogens (10). These areas, including wetlands, marshes and tidal zones, are often situated near urbanized areas (such as harbours), bringing introduced pathogens and vectors in close contact with humans. The knowledge on the larval ecology is important to better assess the areas where certain species can occur and how interventions such as creation of new wetlands, marshes and tidal zones will change mosquito species composition and abundance. This information can be used to design management strategies aiming at reducing the mosquito population with the objective to also reduce the transmission potential in a certain area. Here, we attempted to fill some gaps on knowledge about ecology of mosquito larvae in Flanders. This knowledge is indispensable if adequate control of mosquitoes becomes urgent during outbreaks of arboviruses or of malaria.

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# *Drosophila suzukii* (Diptera: Drosophilidae): A pest species new to Belgium

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The Asian fruit fly ('spotted wing drosophila', *Drosophila suzukii* MATSUMURA, 1931) has been detected for the first time in Belgium in 2011. This species of Asian origin was first reported in North America in 2008 and in Europe in 2009 (1, 2, 3). *D. suzukii* has become a notorious pest in North America, causing severe losses in fruit production (4). In Europe, only a few reports of considerable crop damage from *D. suzukii* can be found (1, 5). Here we discuss the arrival of *D. suzukii* in Belgium and give recommendations for its identification and pest management.

On 21 September, 2011, a single male of the spotted wing drosophila, *Drosophila suzukii*, was captured in Ostend, Belgium (altitude 4 m ab. s. l.). This highly fecund species differs from most other members of the Drosophilidae in its ability to infect healthy ripening fruit instead of overripe, rotting fruit (1). *D. suzukii* can infect healthy soft-bodied fruit such as blueberries, blackberries, raspberries, cherries, or strawberries, and can even infect hard fruit such as apples and pears (4). After infestation, secondary parasites and invaders, such as *Drosophila melanogaster,* Nitidulidae*, Botrytis* and *Rhizopus* spp. often contribute to further fruit deterioration. These characteristics make *D. suzukii* an economically important pest species that requires management to the full extent possible.

The first reported detection of *D. suzukii* in Europe was in 2008 in Rasquera, Spain (1). Until late 2011, European detection of *D. suzukii* had been limited to dry Mediterranean climates. Now, together with observations in Germany, also in autumn 2011 (6, 7), the discovery in Belgium is of great importance since these are the first records of the species in the more temperate northwest-European regions. Ecological simulations seem to indicate that those Mediterranean conditions are not optimal for the growth of *D. suzukii* (2). Up to now, the only report of crop damage in Europe due to *D. suzukii* was the publication of LEE et al. (2011), who reported crop destructions of up to 80% in Trentino, Italy. However, HAUSER (2009) suggested that *D. suzukii* could develop into a serious pest in the more humid northwest European areas.

Several reports of *D. suzukii* in Europe can be found on the Internet, but published literature is scarce. After its detection in 2008 in Spain, the species was found in 2009 in several southern European areas: southern France (Montpellier, Languedoc-Roussillon, Provence-Alpes-Côte d'Azur, Corsica, Alpes-Maritimes), Spain (Barcelona) and Italy (Piemonte, Trentino, Tuscany).

Unconfirmed records from Portugal were made available through a media report (1, 9, 10). In 2010, the fly was reported in Slovenia (11). The first northern European report was from HENDRICH et al. (2012), who mentioned first sightings of the species in the German provinces Baden-Württemberg, Bavaria and Rhineland-Palatinate in 2011. Recently, *D. suzukii* was

detected in the Swiss locations of Graubünden and Tessin (12), and in Austria (11).

The species seems to spread rapidly across the European mainland in a similar manner to the extensive and rapid spread across North America. Adults are highly mobile within a local area, but long-distance dispersal probably occurs via transport of infected fruit. The *D. suzukii* fly detected in Ostend, Belgium could have been imported with infected fruit from the nearby harbour in Zeebrugge. The Belgian authorities are vigilant for the presence of the fly and rely on the Institute for Agricultural and Fisheries Research (ILVO, Plant Sciences Unit - Crop Protection research area) for information about the presence of this species in Belgium. The Belgian Federal Agency for the Safety of the Food Chain (FASFC) will perform a quickscan monitoring program in 2012 to determine whether *D. suzukii* is settling in Belgium. In addition, the Belgian fruit research center (pcfruit) plans to investigate the potential spread and phytosanitary impact of *D. suzukii* in the major fruit growing regions of Belgium (13). Traps with attractants to gather *D. suzukii* were put on the market in February 2012 (14). This monitoring is crucial because quick intervention can prevent crop damage and further spread of the pest. Damage can be prevented by immediately removing the flies and physically destroying infested fruit as fast as possible (4).

The spotted wing drosophila is easy to identify. Males can be identified based on the spots in the tip of their wing and the two sets of tarsal combs. Females have a long and narrow ovipositor with many dark sclerotized teeth (15). Further information on identification can be found in Hauser (2011). In Figures 1 & 2, the habitus of the male and the typical tarsal combs are illustrated.

All observations of *D. suzukii* (even in cases of doubt) should be reported to the corresponding author listed above, to the authorities, and to the research centers as quickly as possible.

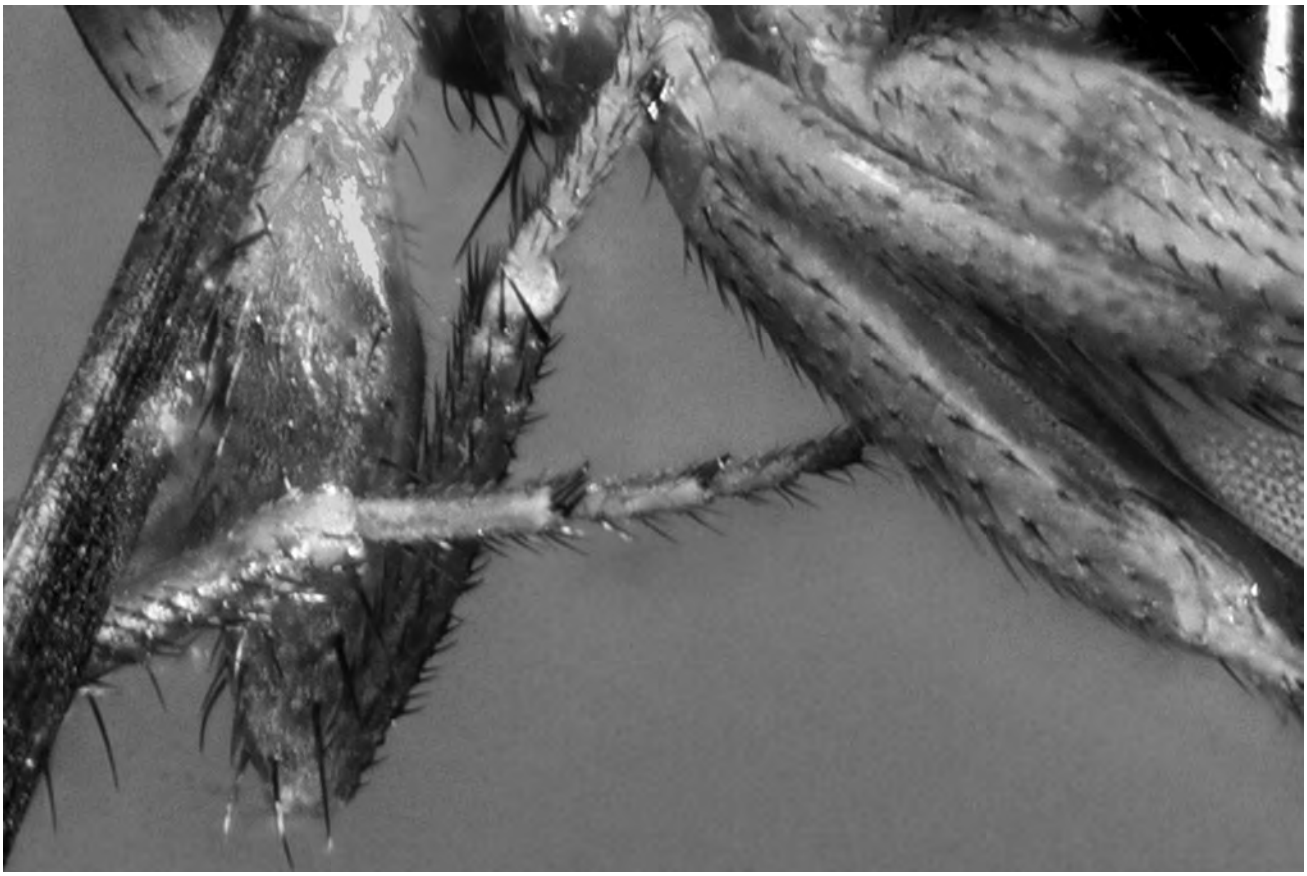

Fig. 1. – Detail of the male tarsal combs.

Fig. 2. – Habitus of the Belgian specimen of *Drosophila suzukii*

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# An easy, cheap computerized method to assess two-dimensional trajectory parameters

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KEY WORDS: angular speed, cemetaries, drugs, linear speed, orientation.

Movement is essential for the survival of mobile organisms. Its study can help to determine taxonomic status (1), to isolate pheromones (2) and to understand biological mechanisms (3). It can also provide information on the health, physiological state and motivation of animals. However, it has rarely been rigorously quantified. We devised a manual method in 1973 (4) and computerized it in 1991 (5) but, despite its continuing use (e.g. 6), this processing became obsolete due to the evolution of computers. Plenty of modern programs exist (7 and references therein, 8, 9 and references therein) but require expensive equipment, take a long time and are generally appropriate for only one kind of assessment. We developed a user-friendly, cheap method that allows simultaneous assessment of orientation, linear speed and angular speed of any moving agent.

This software was tested on the ant *Myrmica rubra*, in a colony being maintained in the laboratory (Fig. 1A). Stimuli presented to the foragers were pieces (1 $cm^2$) of white paper and isolated heads of congeners, which emit the species' alarm pheromone.

Ant trajectories were manually recorded, using a water-proof marker pen, on a glass slide set over the ants' foraging area. They were then traced onto transparent polyvinyl sheets, which stuck to the screen of any PC (Fig. 1B). The trajectories could then be analyzed using the newly elaborated software installed on the PC:

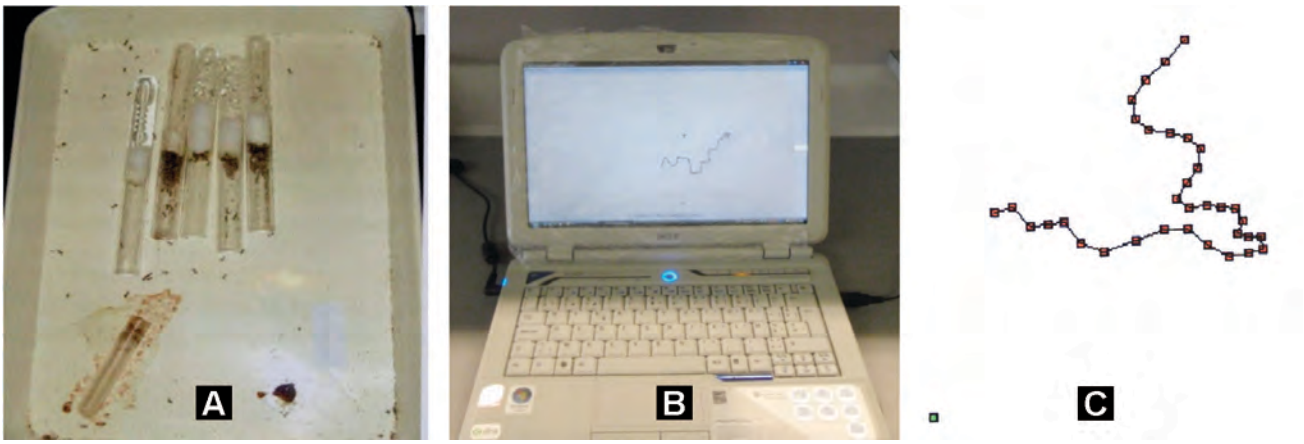
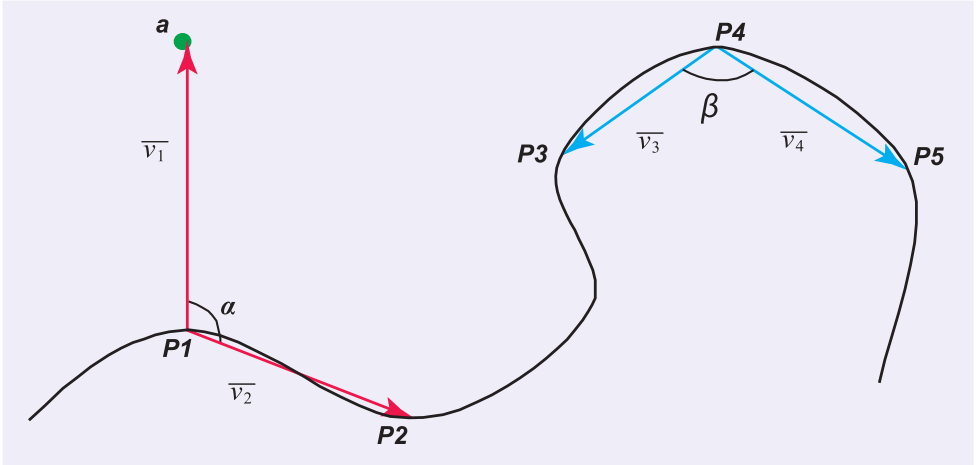



Fig. 1. – Three steps in the computerized analysis of trajectories. **A**: ants are kept in the laboratory in artificial nests. Trajectories are recorded on a glass slide set above the ants' tray and are then traced on a polyvinyl sheet. **B**: this sheet is stuck to the screen of a PC. Each trajectory is entered using a mouse. **C**: the updated software visualizes each trajectory and quantifies its orientation, linear and angular speed.

1. The distance between two points on the screen, initially assessed in pixels, is converted into a metric unit using a dialog box, for both the X- and Y-axes.
2. The successive points of the trajectory are entered by clicking with the mouse, which visualizes, in red, the trajectory on the screen (Fig. 1C). The point towards which the moving agent was expected to go is then located, in green, on the screen (Fig. 1C).
3. The user then states that the trajectory entering is finished and, after that, he/she enters, in a window, the total time spent by the moving agent to move along its trajectory.
4. Validating the last operation starts the calculation, by the newly-elaborated software, of the three following variables (Fig. 2). The orientation (O) of the moving agent towards a given point of the environment is the sum of the angles, measured at each successive point of the registered trajectory, made by the segment 'point i of the trajectory – given point' and the segment 'point i – point i + 1' divided by the number of measured angles. This variable can be measured in angular degrees, for instance. The linear speed (V) of the agent is the length of its trajectory divided by the time spent moving along this trajectory. It can be measured in mm/s, for instance. The angular speed (S) (i.e. the sinuosity) of the animal's trajectory is the sum of the angles, measured at each successive point of the trajectory, made by the segment 'point i – point i – 1' and the segment 'point i – point i + 1', divided by the length of the trajectory. This variable can be measured in angular degrees/cm, for instance.
5. The required calculated values appear on the screen of the PC, the entire operation lasting 20-30 sec. The user can then 'shut the program' or 'begin again', directly entering a new trajectory.




a = a given point in the environment

**V**: distance / time

$$\mathbf{O:}\begin{cases}\overline{v_1} = (a_x - p1_x, a_y - p1_y)\\ \overline{v_2} = (p2_x - p1_x, p2_y - p1_y)\end{cases} \qquad \mathbf{S:}\begin{cases}\overline{v_3} = (p3_x - p4_x, p3_y - p4_y)\\ \overline{v_4} = (p5_x - p4_x, p5_y - p4_y)\end{cases}$$

$$\overline{v_1}\cdot\overline{v_2} = \|\overline{v_1}\|\|\overline{v_2}\|\cos\alpha \qquad \overline{v_3}\cdot\overline{v_4} = \|\overline{v_3}\|\|\overline{v_4}\|\cos\beta$$

$$\rightarrow \alpha = \cos^{-1}\left(\overline{v_1}\cdot\overline{v_2}\,/\,\|\overline{v_1}\|\|\overline{v_2}\|\right) \qquad \rightarrow \beta = \cos^{-1}\left(\overline{v_3}\cdot\overline{v_4}\,/\,\|\overline{v_3}\|\|\overline{v_4}\|\right)$$

$$O = \Sigma_i\,\alpha_i / number\ of\ angles \qquad S = \Sigma_i\,\beta_i / distance$$

Fig. 2. – Mathematical reasoning underlying the quantification of the orientation (**O**), linear speed (**V**) and angular speed (**S**) of a trajectory. The three variables are defined in the text.

Table 1

Comparison of the manual **(M)** and the computerized **(L)** method. Ten ant trajectories obtained in the presence of a blank paper (control) and of an isolated congener's head (test) were analyzed and the difference between the two methods was evaluated. Differences are less than the experimental errors. O=orientation (angular degrees), V=linear speed (mm/sec), S=angular speed (angular degrees/cm).

| Control | | | | | | | | | | | mean | difference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| O | **M** | 74.6 | 124.2 | 101.9 | 119.3 | 105.0 | 114.0 | 86.8 | 106.1 | 57.3 | 101.1 | 99.8 | 6.5% |
| | **L** | 70.4 | 113.8 | 98.3 | 118.1 | 91.5 | 109.7 | 89.9 | 101.6 | 44.5 | 97.2 | 93.5 | |
| V | **M** | 11.0 | 7.0 | 8.0 | 8.0 | 12.5 | 10.0 | 11.5 | 9.0 | 10.0 | 10.0 | 9.6 | 12% |
| | **L** | 11.6 | 6.8 | 10.3 | 6.0 | 10.8 | 10.6 | 9.5 | 10.1 | 9.3 | 10.3 | 8.5 | |
| S | **M** | 137 | 156 | 183 | 63 | 138 | 138 | 155 | 253 | 119 | 214 | 162 | 5% |
| | **L** | 138 | 149 | 182 | 62 | 153 | 149 | 166 | 225 | 113 | 201 | 154 | |

| Test | | | | | | | | | | | mean | difference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| O | **M** | 53.3 | 38.0 | 46.0 | 39.2 | 33.3 | 42.0 | 60.9 | 20.0 | 60.0 | 31.3 | 42.4 | 4.6% |
| | **L** | 55.2 | 36.2 | 43.3 | 37.0 | 31.5 | 44.9 | 64.3 | 17.2 | 52.3 | 22.9 | 40.5 | |
| V | **M** | 12.0 | 16.0 | 18.0 | 20.0 | 18.0 | 22.0 | 24.0 | 17.0 | 24.0 | 18.0 | 18.9 | 3% |
| | **L** | 13.4 | 15.4 | 18.2 | 17.2 | 16.6 | 19.2 | 21.9 | 16.5 | 26.7 | 17.7 | 18.3 | |
| S | **M** | 109 | 100 | 106 | 109 | 106 | 99 | 115 | 145 | 105 | 157 | 115.1 | 0.6% |
| | **L** | 93 | 109 | 73 | 126 | 87 | 111 | 122 | 150 | 105 | 183 | 115.8 | |

Table 2

Assessment of the linear (V) and angular speed (S) of *Tribolium castaneum* and of *Paramecium caudatum* under control and experimental conditions. *T. castaneum* was observed directly, like the ants, while *P. caudatum* was observed under a stereomicroscope (Mag. = 23 X), this requiring a unit adaptation. N = number of individuals observed; results of non-parametric $\chi^2$ tests between control and experiments: P= level of probability; NS = difference not significant at P = 0.05. An activated GSM had an impact on the observed animals.

| *T. castaneum* | N | V (mm / sec) | S (angular degrees / cm) |
|---|---|---|---|
| Control | 42 | 5.2 (4.6 - 5.8) | 150 (120 - 183) |
| + GSM on | 31 | 3.8 (3.2 - 4.4) P < 0.001 | 398 (343 - 469) P < 0.001 |
| + GSM off | 29 | 5.1 (4.7 - 6.2) NS | 174 (145 - 220) NS |
| *P. caudatum* | N | V (mm / sec) | S (angular degrees / mm) |
| Control | 23 | 0.63 (0.57- 0.67) | 179 (138 - 200) |
| + GSM on | 34 | 0.50 (0.39 - 0.58) P < 0.001 | 465 (340 - 534) P < 0.001 |
| + GSM off | 32 | 0.66 (0.59 - 0.74) NS | 172 (117 - 196) NS |

The manual and the computerized methods give identical results (Table 1), but the computerized one is 30 times faster and therefore allows analysis of many more trajectories, and is more precise, human errors being avoided.

The newly-computerized method was then used to make five assessments, and was thus tested.

1. Trajectories of the beetle *Tribolium castaneum* were successfully analyzed under normal conditions, near a switched-on mobile phone (GSM) and near a switched-off GSM (Table 2). The new method is particularly applicable to small moving animals. Note the effect of an activated GSM on the insects' movement.
2. Trajectories of the protozoan *Paramecium caudatum* were analyzed under normal conditions, near a switched-on GSM and near a switched-off GSM (Table 2). A camera lucida was applied to the stereomicroscope under which *P. caudatum* were set. The new method allows analysis of the movement of any microscopic agent in this manner. Note, once more, the effect of an electromagnetic field on living organisms.
3. Pieces of white paper (1 $cm^2$) were deposited for 8 days on ant cemeteries (Fig. 3A) and were then presented to foragers whose movement was analyzed using the described method (Table 3). The foragers were not attracted by the papers but their angular speed considerably increased. Ants transporting corpses move thus randomly away from their nest and in a sinuous increasingly slowing-down pattern as they come nearer to a cemetery. They finally stop there and drop the corpses. While returning then to their nest, they deposit their trail pheromone along a short distance (personal observation), which explains the ethological effect of cemetery sites on the ants. The new computerized method thus provided, in a few minutes, an explanation for the presence of ant cemeteries, on given places, far from the nests.
4. *Myrmica* ants are attracted by their specific alarm pheromone contained in the head of workers (Table 4). Cross-tests using isolated heads of known and unknown ants (f.i. newly collected) (Fig. 3B) followed by analysis of the numerous recorded ant trajectories enable recognition of an unknown (f.i. collected) species. Such a long process can be efficiently performed only by using this rapid computerized method. Such taxonomic recognition of closely related species can be

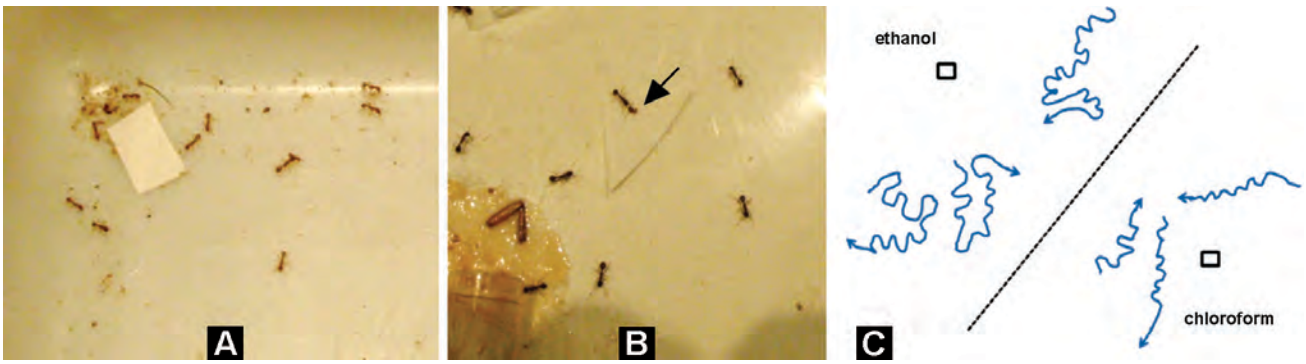



Fig. 3. – Three illustrated uses of the method. **A:** pieces of paper were deposited on ant cemeteries and then presented to foragers. They were not attractive to the ants but decreased their angular speed. They thus may be impregnated with trail pheromone deposited by ants leaving the cemeteries sites. **B:** isolated heads of three ant species were presented to foragers of these species to see if such cross tests can help recognizing unknown species. Here, the head of an individual of *M. sabuleti* (pointed by an arrow) is presented to workers of *M. rubra*, which are not attracted by the non-specific stimulus. Cross tests and assessments using our method can thus help discriminating between species. **C:** trajectories of ants moving near a small amount of ethanol or chloroform. Ethanol increased the ants' linear and angular speed while chloroform decreased their linear speed. Simple ethological tests together with our software-based method can help detect minute amounts of drugs in samples.

Table 3

Locomotion of *Myrmica sabuleti* foragers in front of their cemeteries. Blank pieces of paper or paper deposited for 8 days at cemeteries were presented to foragers. The orientation towards the paper (angular degrees), the linear speed (mm/sec) and the angular speed (angular degrees/cm) of 60 or 30 (= N) foragers were assessed using our software. The distributions of the values obtained for each two stimuli were compared using the non-parametric $\chi^2$ test. P = level of probability; NS = difference not significant at P = 0.05.

| variable | untreated paper | paper deposited at cemeteries | statistics |
|---|---|---|---|
| Orientation | N = 60 89.3 (66.6 – 105.3) | N = 30 91.1 (75.7 – 107.0) | NS |
| Linear speed | N = 60 12.8 (11.8 – 14.7) | N = 30 11.1 (9.2 – 14.8) | P < 0.05 |
| Angular speed | N = 60 183 (147 – 211) | N = 30 223 (211 – 245) | P < 0.001 |

Table 4

Cross-tests between three *Myrmica* species, using isolated worker heads presented to foragers. The orientation (O; angular degrees) towards the head, the linear speed (V; mm/sec) and the angular speed (S; angular degrees/cm) of 10 foragers were assessed each time, using our software. Ants clearly oriented themselves only towards isolated heads of their own species. Cross-tests, together with our computerized method, are thus helpful for taxonomic purposes.

| Species whose head was presented | Tested species *M. rubra* | *M. ruginodis* | *M. sabuleti* |
|---|---|---|---|
| *Myrmica rubra* | O 44.7(42.5-52.0)<br>V 24.6(24.1-26.3)<br>S 77 (75-80) | O 105.5(86.2-119.8)<br>V 19.3(16.8-20.6)<br>S 91 (76-106) | O 81.4(69.5-96.7)<br>V 17.2(16.2-17.9)<br>S 130 (121-150) |
| *Myrmica ruginodis* | O 82.8(76.9-89.7)<br>V 19.6(18.9-20.8)<br>S 116 (111-148) | O 34.1(31.4-46.0)<br>V 29.8(27.6-32.8)<br>S 79 (63-91) | O 93.9(82.9-101.5)<br>V 22.5(20.2-24.9)<br>S 116 (110-127) |
| *Myrmica sabuleti* | O 107.2(95.7-118.2)<br>V 19.6(17.6-22.5)<br>S 160 (147-170) | O 93.6(74.8-118.2)<br>V 20.0(17.3-20.7)<br>S 121 (97- 134) | O 44.4(35.1-57.3)<br>V 22.8(19.3-24.1)<br>S 126 (106-143) |

Table 5

Response of *Myrmica sabuleti* workers to ethanol and chloroform. 10 µl of differently-concentrated solutions of these substances were presented to foragers and the locomotion of 10 of them was assessed using the here related software. The concentration (%) is given in the first column; the quantity (µl) presented, in the second one. O = orientation towards the stimulus, angular degrees; V = linear speed, mm/sec; S = angular speed, angular degrees/cm. $\chi^2$ tests between results for 'pure water' and 'substances': P = level of probability, * = P < 0.05 or 0.02, ** = P < 0.001, otherwise = result non significant at P = 0.05.

| Concentration | Quantity | O | V | S |
|---|---|---|---|---|
| Pure water | | 66.3(61.2-71.7) | 14.5(13.7-15.7) | 142(133-153) |
| Ethanol | | | | |
| 0.001 | 0.0001 | 80.9(70.3-108.8) | 18.0(15.6-21.2) * | 167(154-194) * |
| 0.01 | 0.001 | 78.8(68.3-89.3) | 20.5(18.1-22.1) ** | 194(181-203) ** |
| 0.1 | 0.01 | 81.1(61.7-105.8) | 21.3(16.7-23.2) ** | 221(213-232) ** |
| 1 | 0.1 | 101.3(69.5-109.7) | 24.6(21.6-26.9) ** | 216(197-241) ** |
| 10 | 1 | 77.9(67.3-91.8) | 25.4(22.3-28.3) ** | 218(204-228) ** |
| Chloroform | | | | |
| 0.0001 | 0.00001 | 77.3(60.7-93.2) | 12.8(12.2-14.2) | 190(173-224) ** |
| 0.001 | 0.0001 | 97.4(87.9-111.2) | 9.7(7.3-11.9) ** | 212(161-239) ** |
| 0.01 | 0.001 | 88.5(80.4-102.0) | 8.8(8.1-9.2) ** | 279(266-291) ** |
| 0.1 | 0.01 | 85.1(65.2-99.2) | 8.1(6.8-9.8) ** | 275(223-297) ** |

extended to any animals that have specific pheromonal secretions. It can be used as an aid to morphological or genetic determination. This technique should be applied, for instance, to related bumblebee species (10), virgin females responding only to the pheromonal secretion of conspecific males.

5. *Myrmica* ants react to ethanol by increasing their linear and angular speed (Fig. 3C), and do so down to 0.0001 µl of ethanol, which corresponds to an aqueous solution of 0.001% (Table 5). These ants also react to chloroform, but by decreasing their linear speed (Fig. 3C), this occurring down to a presentation of 0.00001 µl of chloroform, e.g. an aqueous solution of 0.0001%. For revealing these kinetic reactions, many trajectories must be analyzed, and this can be done, in a short time, only by using this rapid, simple method. So, using this method, *Myrmica* ants can be used to detect small amount of any given drugs in collected material.

In conclusion, RODUIT (11) wrote: 'no universal solution exists for the analysis of trajectories'. This is true when the solution requires highly technical equipment, sophisticated software and many conditions for being used. On the contrary, a simple method – requiring cheap material, easy-to-use software and having no conditions for being used – may be universal or, at least, used in a first step to check if it may be promising to use more onerous methods. The user-friendly system we have here related is such a simple method. It requires no program license and can be used by many persons at the same time. It is thus competitive with other more sophisticated methods. The software, labeled OVS, will be available on the website of the journal as soon as the present paper is published.

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# Larval mantis shrimp *Rissoides desmaresti* (Risso, 1816) (Stomatopoda) in the Belgian part of the North Sea

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KEY WORDS: *Rissoides desmaresti* larvae, mantis shrimp, Stomatopoda, Belgian part of the North Sea.

The mantis shrimp *Rissoides desmaresti* (Risso, 1816) is a stomatopod crustacean (Stomatopoda: Squillidae), native to the Mediterranean Sea and the North East Atlantic from the Southern North Sea to the coasts of Madeira (Portugal) (1, 2, 3).

Adult *R. desmaresti* are benthic and burrow in the sediment (4). They occupy sub-littoral habitats to a depth of 75-80 m (5) and can reach lengths up to 97 mm (6). Adults are fast and efficient ambush predators that use their two-toothed, raptorial forelimbs (2nd thoracopods) as a spear to capture small fish and shrimps (7). They are preyed upon by demersal fish, such as tope *Galeorhinus galeus* (Linnaeus, 1758) and bull-rout *Myoxocephalus scorpius* (Linnaeus, 1758) (6, 8).

The larvae of *R. desmaresti* (Fig. 1) are planktonic, have a total body length of 3.6 to 22.5 mm, and also possess strong raptorial appendices, which are mainly used to prey upon larvae and eggs of echinoderms and molluscs (9, 10).

Both adult and larval specimens of *R. desmaresti* have been reported infrequently in the Southern North Sea and English Channel region (8, 11). In Belgian waters, adults have so far never been recorded (12). However, Stomatopoda larvae were collected by G. Gilson during the European ICES (International Council of the Exploration of the Sea) campaigns between 1902 and 1913 (Gilson collection, largely preserved at the Royal Belgian Institute for Natural Sciences (RBINS)


Fig. 1. – Picture of the 6th stage megalopa larva of *Rissoides desmaresti* (specimen 1).

in Brussels, Belgium). Several specimens that were identified as *Erichthus* larvae were re-identified in the 1960s as larvae of *Squilla desmaresti* (11), nowadays renamed to *Rissoides desmaresti* (13). An overview of these findings is shown in Figure 2. Some other specimens could not be re-identified as they were absent from the Gilson collection, but are likely to be larvae of *R. desmaresti*. The latter are presented as '*Erichthus*' observations in Figure 2. Larvae of *R. desmaresti* have been found all over the Belgian part of the North Sea (BPNS) area. However, since the early 1900s, no more recordings of *R. desmaresti* larvae were made or could be uncovered for the BPNS, not even in more recent hyperbenthic and zooplanktonic studies performed in this area (14, 15).

Almost a century later, in August and September 2011, five larval *R. desmaresti* specimens were caught during zooplankton sampling campaigns in the BPNS on board RV Zeeleeuw. Four larvae were found at monitoring station W09 (N 51°45' E 2°42') situated north of 'Hinderbanken' and one at 'Thornton bank' monitoring station W07tris (N 51° 31.72' E 2° 52.44') (Fig. 2). A WP3 net (mesh size 1000 µm, Ø 1 m) was employed to collect the zooplankton samples. The net was trawled at a speed of approximately three knots, filtering the water column four times from surface to bottom in an undulating haul. Zooplankton samples were preserved in 4% buffered formaldehyde and analysed in the laboratory, using a stereomicroscope.

The larval morphology of *R. desmaresti* can easily be distinguished from another Stomatopoda species *Platysquilla eusebia* (Risso, 1816) that is also found in the North Sea, by comparing the shapes of the carapax and telson (9, 11).

During larval development nine megalopa stages can be morphologically distinguished (9). The three specimens collected in August could be allocated to the 6th and 7th stage, the two specimens caught in September to the 8th stage. Examined identification characteristics are listed in Table 1.

The larvae that were re-identified from the Gilson collection were also caught in August and September, but belonged to different developmental stages, ranging from 2nd megalopa to postlarval stage (Table 2). The duration of larval development in *R. desmaresti* has not thoroughly been investigated yet. However, there are similarities with other Squillidae, in particular *Squilla mantis* (Linnaeus, 1758). In late autumn and winter, female mantis shrimp prepare for reproduction, but spawning only happens in spring (mid-March – mid-April) (6, 9). After a ten-week incubation period (as in *S. mantis*), stage 1 megalopa larvae of *R. desmaresti* should be present in the water in June or July. Stage 8 and 9 larvae should show up in the plankton between August and October (8-12 weeks later, just as in *S. mantis*), which is consistent with our findings for *R. desmaresti* (Table 2). Hereafter,

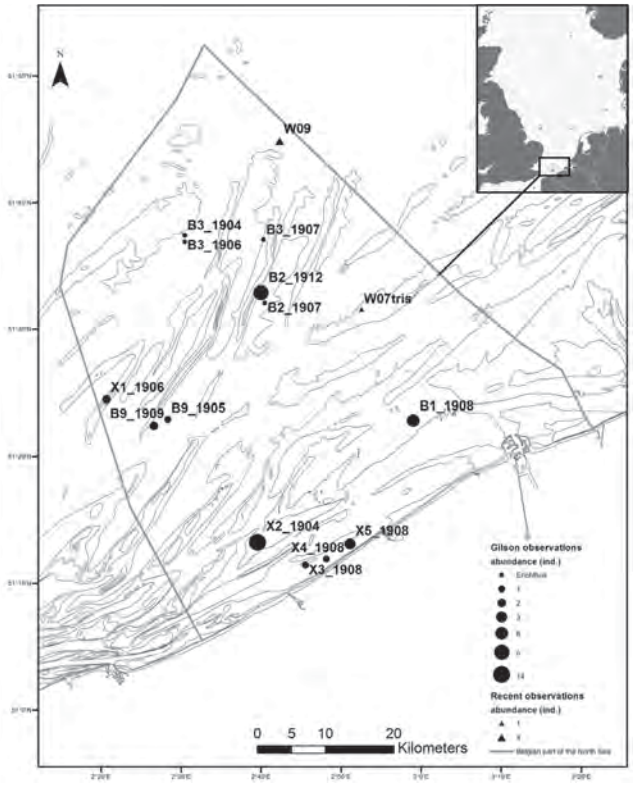


Fig. 2. – Spatial distribution of larval *Rissoides desmaresti* in the Belgian part of the North Sea. Triangles (▲) indicate recent observations; circles (●) indicate observations by Gilson from the early 1900s. Note: Coordinates of stations labeled 'X' are estimations based on descriptions in van der Baan & Holthuis (11).

TABLE 1

Examined identification characteristics for the five collected specimens (verified with GIESBRECHT (9)), A= antenulla, B= basis, I= ischium, T1= 1st thoracopod or 'cleaning leg', T2= 2nd thoracopod or raptorial leg, T3 – T5= 3rd – 5th thoracopod, na= not applicable, + = present, - = absent.

| Characteristics | Spec 1 | Spec 2 | Spec 3 | Spec 4 | Spec 5 |
|---|---|---|---|---|---|
| Location | W07tris | W09 | W09 | W09 | W09 |
| Date of collection | 3 Aug 2011 | 3 Aug 2011 | 3 Aug 2011 | 2 Sep 2011 | 2 Sep 2011 |
| Filtered volume ($m^3$) | 85 | 242 | 242 | 483 | 483 |
| Density ($ind/m^3$) | 0.012 | --------0.008-------- | | --------0.004-------- | |
| Length (rostrum-telson) (mm) | 10.7 | 11.4 | 10.8 | 17.0 | 17.0 |
| # articles dorsal flagellum of A | 2 | 2 | 3 | 8 | 8 |
| # articles ventral flagellum of A | 1 | 1 | 2 | 4 | 4 |
| Ratio width and length telson | na | na | 4:5 | na | na |
| Ratio B + I of T3 and B of T2 | 2:7 | 2:7 | 1:2 | >3:4 | >3:4 |
| Gills of T3 | na | na | + | na | na |
| Gills of T4 | na | na | + | na | na |
| Gills of T5 | na | na | - | na | na |
| Gills T1 equal in size as gills T2 | na | na | na | yes | yes |
| Larval development stage | 6 | 6 | 7 | 8 | 8 |

the larva undergoes metamorphosis (four postlarval stages were described by GIESBRECHT (9)), which results in a pubescent adult living in and on the sediment (duration approximately 2-3 months) (6).

It is unclear how the larvae of *R. desmaresti* arrived in the BPNS. Adult Stomatopoda have never been observed in the BPNS despite regular benthic monitoring campaigns with Van Veen grabs and an 8 m shrimp trawl (mesh size 20 mm in the cod end) since the late 1970s (12). Benthic specialists were addressed, but none of them could confirm an observation in the BPNS. However, adults were recently observed at the east, south (including English Channel area) and west coast of the UK by divers and in beam trawl and grab samples (4, 8, 16). There are a few observations in the Dutch part of the North Sea from the early 1900s (11) and a few recent unpublished observations. The Southern North Sea is known as the northern boundary of the distribution range for *R. desmaresti*. The northernmost sighting of an adult was offshore from the Dutch Wadden islands (N 53°42' E 3°52') on 31 January 1963 (11).

Since dominant surface currents run in a north easterly direction, larvae might be transported to the BPNS and beyond from populations in the English Channel and the south coast of the UK (17). Increase in sea water temperature due to global warming might favour this larval transport and survival.

The absence of adult Stomatopoda in the BPNS is probably also related to the lack of suitable habitat. Adults require a particular sediment

TABLE 2

Overview of larval stages of *Rissoides desmaresti* found in the Belgian part of the North Sea (M = megalopa, PL = postlarva), recent observations are marked in bold.

| Station | Date | Larval stages | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M1 | M2 | M3 | M4 | M5 | M6 | M7 | M8 | M9 | PL |
| X2_1904 | 29/08/1904 | | | | | | | 14 | | | |
| B9_1905 | 25/08/1905 | | | | | | | 1 | | | |
| X1_1906 | 23/08/1906 | | | 1 | | | 1 | | | | |
| B1_1908 | 25/08/1908 | | | | | | 6 | | | | |
| X5_1908 | 1/09/1908 | | | | | | | | | 2 | 1 |
| X3_1908 | 16/09/1908 | | | | | | | | 1 | | |
| X4_1908 | 23/09/1908 | | | | | | | | | 1 | |
| B9_1909 | 22/08/1909 | | | | 1 | | | 1 | | | |
| B2_1912 | 25/08/1912 | | | | | 9 | | | | | |
| **W07tris** | **6/08/2011** | | | | | | **1** | | | | |
| **W09_Aug** | **7/08/2011** | | | | | | **1** | **1** | | | |
| **W09_Sept** | **8/09/2011** | | | | | | | | **2** | | |

composition (a mixture of mud, sand and gravel) to construct a U-shaped burrow, while they avoid sites with either high mud concentration (> 70%) or sandy sediments with very low mud concentrations (≤ 2%) (4). The BPNS is characterised by mixed sediments, but only the nearshore area (overlapping with the *Abra alba* benthic community (18, 19)) contains enough mud to construct cohesive burrows (20). Together with disturbance by ubiquitous demersal fishing activities, the current lack of a proper gravel concentration in the sediment mixture probably prevents the settlement of stable *R. desmaresti* populations.

In conclusion, this manuscript describes five new recordings of larvae of the mantis shrimp *Rissoides desmaresti* in the Belgian part of the North Sea, which are the first recordings since the early 1900s. The species *R. desmaresti* and the order Stomatopoda can now be added to the Belgian marine species list (12). The larvae were most probably transported with the currents through the English Channel, possibly favoured by global sea water temperature increase.

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