Department of Theoretical Biology

universität wien

MASTERARBEIT

Titel:

3D Atlas über die Organogenese von *Euprymna scolopes* (Cephalopoda: *Sepiolidae*) und die Organlage in Adulten

A 3D Developmental Atlas of *Euprymna scolopes*

(Cephalopoda: *Sepiolidae*)

Verfasst von

Claudia Klimpfinger BSc

Angestrebter akademischer Grad:

Master of Science (MSc)

Wien, 2015

Studienkennzahl lt. Studienblatt: A 066 831

Studienrichtung lt. Studienblatt: Masterstudium Zoologie UG2002

Betreut von: Ao. Univ.-Prof. DDr.Gerd Müller

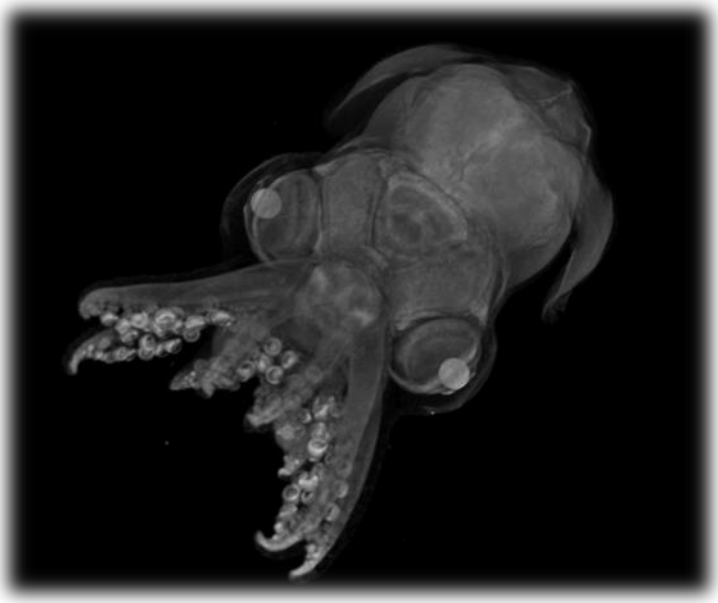
Department of Theoretical Biology

universität wien

MASTER THESIS

Title:

A 3D Developmental Atlas of *Euprymna scolopes* (Cephalopoda: *Sepiolidae*)



Author:
Claudia Klimpfinger BSc

Targeted academic degree:
Master of Science (MSc)

Vienna, 2015

Characteristic number of study: A 066 831
Field of study: Zoology
Adviser: Ao. Univ.-Prof. DDr.Gerd Müller
Co-Advisor: Dr. Brian Metscher

Index of contents

Abstract

The Hawaiian bobtail squid *Euprymna scolopes* Berry, 1913, belongs to the sepiolid family and is an emerging model organism for developmental studies in decabrachiate cephalopods and animal-bacteria symbioses. However, the anatomy and the development of indiviual organ systems have received only very little attention and therefore the aim of this project is to prepare a closer view on *Euprymna scolopes* organogenesis and the organ topography of the adults. This project is an amendment to former papers from Lee et al. and Arnold et al. and shows the development of organ systems in colored 3D images including a video of the adult. This project produced a 3D image database of the anatomy and the development of the organ systems of *E. scolopes*, containing pictures of the stages 19-30 (whereas stage 30 represents the hatchling) and of two adult individuals (male and female) as well as high-resolution size-calibrated microtomographic (microCT) images. The final work contains a series of 3D images – one of every stage of development and of the adult – and also metadata of each stage plus a video of the adult male. These will be archived along with the original microCT stacks. The pictures are published with open-access, so everyone interested in this squid is able to work with this basic information. Therefore the language used in this paper has been chosen intentionally to be non-technical, so that everyone can understand the work easily.

Keywords

Euprymna scolopes, microCT, organogenesis, 3D online atlas, Cephalopoda

Kurzfassung

Der Hawaiianische Zwergtintenfisch *Euprymna scolopes* Berry, 1913, gehört zur Familie der Zwergtintenfische (Sepiolidae) und ist ein potentieller, aufkommender Modelorganismus für Entwicklungsstudien von zehnarmigen Cephalopoden sowie von Tier-Bakterien-Symbiosen und deren Fortbestand während der gesamten Lebensdauer der Tiere. Bis heute wurde der Anatomie und der Entwicklung der Organsysteme eher wenig Aufmerksamkeit zu teil. Daher ist es das Ziel dieses Projektes eine genauere Darstellung der Organogenese von *Euprymna scolopes* sowie der Topographie der Organe im adulten Tier zu erarbeiten. Diese Arbeit ist eine Ergänzung zu früheren Publikationen von Lee et al. und Arnold et al. und zeigt die Entwicklung der Organsysteme in farbigen 3D Bildern und Videos. Als Resultat dieser Masterarbeit ging eine 3D Atlas Datenbank der Anatomie und der Organentwicklung von *E. scolopes* mit hoch auflösenden, größen-geeichten microtomographischen (microCT) Bildern der Entwicklungsstadien 19-30 (wobei 30 das Schlüpf-Stadium repräsentiert) und der erwachsenen Tiere, sowie Metadaten und 3D Modellen derselbigen hervor. Auch ein Video des Adulten Männchens ist zusätzlich zu betrachten. Diese Datenbank ist frei zugänglich, damit jeder, der Interesse an diesen Cephalopoden hat, mit ihr arbeiten kann. Die in dieser Arbeit gewählte Sprache ist bewusst einfach gehalten damit jede/r Interessierte die Informationen und Themen leicht verstehen kann.

Schlüsselworte

Euprymna scolopes, MicroCT, Organogenese, 3D online Atlas, Cephalopoda

Introduction

Cephalopods are extraordinary mollusks equipped with a vertebrate-like intelligence and a unique body-construction including a sophisticated buoyancy system for locomotion. Also they are the only mollusks with a closed blood system. Extant cephalopods are organized into two major groups: the nautiloids and the coleoids. While the nautiloids kept their shell complete and on the outside the coleoids have internalized or completely lost their shell. Within those there is another split in the phylogenetic tree into the decapods, which are the ten-armed cephalopods (such as squids and sepia), and the octopods, which are the eight-armed cephalopods (such as octopus and vampyrotheutis) (Kröger et. al. 2011). One of the many species of decapods is the object of this study: *Euprymna scolopes* Berry 1913 [**Fig. 1**].

Euprymna scolopes is a tiny decabrachiate squid from the sepiolid family and is endemic to the shallow coastal waters of the Hawaiian Islands. When it is an adult its mantle reaches a length of about 30 mm (Jereb and Roper, 2005). *E. scolopes* lives in warm, shallow waters at depths of 3cm up to 1 meter (Moynihan, 1983). Juveniles are only found in shallow waters. Adults are also found in shallow coastal waters but sometimes are trawled offshore in depths of about 250 meters (http://www.thecephalopodpage.org/Escolopes.php).

The Hawaiian bobtail squid, as *Euprymna scolopes* is called by its common name, is a rising model organism in research for bacteria-host-symbiosis since its small size, short life-span, rapid growth and year-round availability predestine it for being a model-organism not only for bacteria-host-symbiosis but also for decabrachiate cephalopod development and evolution. Today *E. scolopes* can be obtained continually and can be reared in the laboratory over an entire generation. The embryos and protective chorions are optically clear which facilitates in situ developmental observations. However, this species is best known for its symbiosis with the luminous marine bacterium *Vibrio fischeri*. The Hawaiian bobtail squid lacks an internal shell, possesses a pair of paddle-shaped fins, and has a bioluminescent light organ used in predator avoidance. Until now an exact lifespan has not been established, but after comparing laboratory-reared and field-caught animals the lifespan probably lasts 1 year. The animals have no explicit larval stage: the juvenile hatchlings just look like miniature adults (Lee et. al. 2009c).

Euprymna scolopes is a nocturnal predator that buries itself in the sand during daytime and also secretes mucus which, according to John Shears (1988), is probably used to keep a sand-coat on the dorsal side of its body when rising from the substrate during daytime or in some cases also during nighttime. On the first days after hatching the juveniles feed on their yolk reserves (Lee et. al. 2009c).

The first attempt to describe the development of *Euprymna scolopes* was made by Arnold Singley and Williams-Arnold (1972) by observing wild-caught egg-masses and randomly selecting a few eggs each day. The selected eggs were separated from the white egg capsule and as much jelly as possible to facilitate visibility when looking at them under a dissecting microscope. Arnold's classification of the embryonic stages is based on the prior developmental studies of *Loligo pealei* (Arnold 1972).

Lee et al. started in 2009 a more precise description of the organogenesis of *E. scolopes* using Arnolds work as a basis. They used a single egg-mass under controlled environmental conditions for the description of the development and took about 10 eggs each day to look at the externally visible morphological features that are easily distinguished in either live or freshly fixed embryos under a dissecting microscope. They started at cleavage and went on until hatching with photomicrographs as an aid in the accurate and rapid staging of *E. scolopes*. The aim of their work was a staging series based on easily distinguishable morphological features which should provide a consistent reference for comparisons between independent studies. This staging series should also help avoiding the need to know when fertilization occurred and should allow a correlation of the stage of development with the time of development (Lee et. al. 2009b).

Alexandra Kerbl took an attempt of working on *Euprymna scolopes* with microCT as an extension of histological methods in 2012, concentrating on the development of the central nervous system and the ganglionic system. She detected the first histological evidence of the nervous system at stage 19, as mentioned in previous studies, when some ganglionic placodes already accumulate (Kerbl, 2012). Using microCT Kerbl et al. argued that the poor tissue condition in animals younger than stage 24 made microCT unsuitable for detailed reconstruction of the ganglionic system in these early stages. In the stages older than stage 24 the ganglionic tissue was well visible (Kerbl et al. 2013).

The 3D developmental atlas I am producing within the limits of this Master thesis is intended to provide a new view on the embryogenesis of *Euprymna scolopes* making structures visible that cannot be seen with the dissection microscope. It also is the first attempt of describing the anatomy of *Euprymna scolopes* – excepting the nervous system – with three dimensional tools. The online atlas shows the marked organs added one by one to the basic image-stack. The atlas should facilitate the search for single structures as the 3D pictures generated by the downloaded stack can be turned in different directions and because it is also possible to show one or more specific organs at a time. Besides that the atlas should pique the curiosity of more people to make them more interested in cephalopods. The atlas may also be used as a teaching tool (for schools or university) and since there is no anatomical map for adults until now, this project should fill this gap too.

Another goal of this work is the support of developmental imaging to visualize developmental morphologies and processes better. The reconstructed images can be used to generate quantitative 3D data which then can be used for statistical analysis of 3D forms. In the Department of Theoretical Biology of the University of Vienna microCT scans are used as a new tool for morphological and morphometric studies of patterning and function in embryos. It is a goal of the Department to settle microCT as a standardized method for generating quantitative 3D imaging data for morphometric issues as well as for molecular and genetic patterns (http://theoretical.univie.ac.at/). Recent publications of the staff members of the Department give an insight of the approaches (Herdina et al. 2015; Mayer et al. 2014).

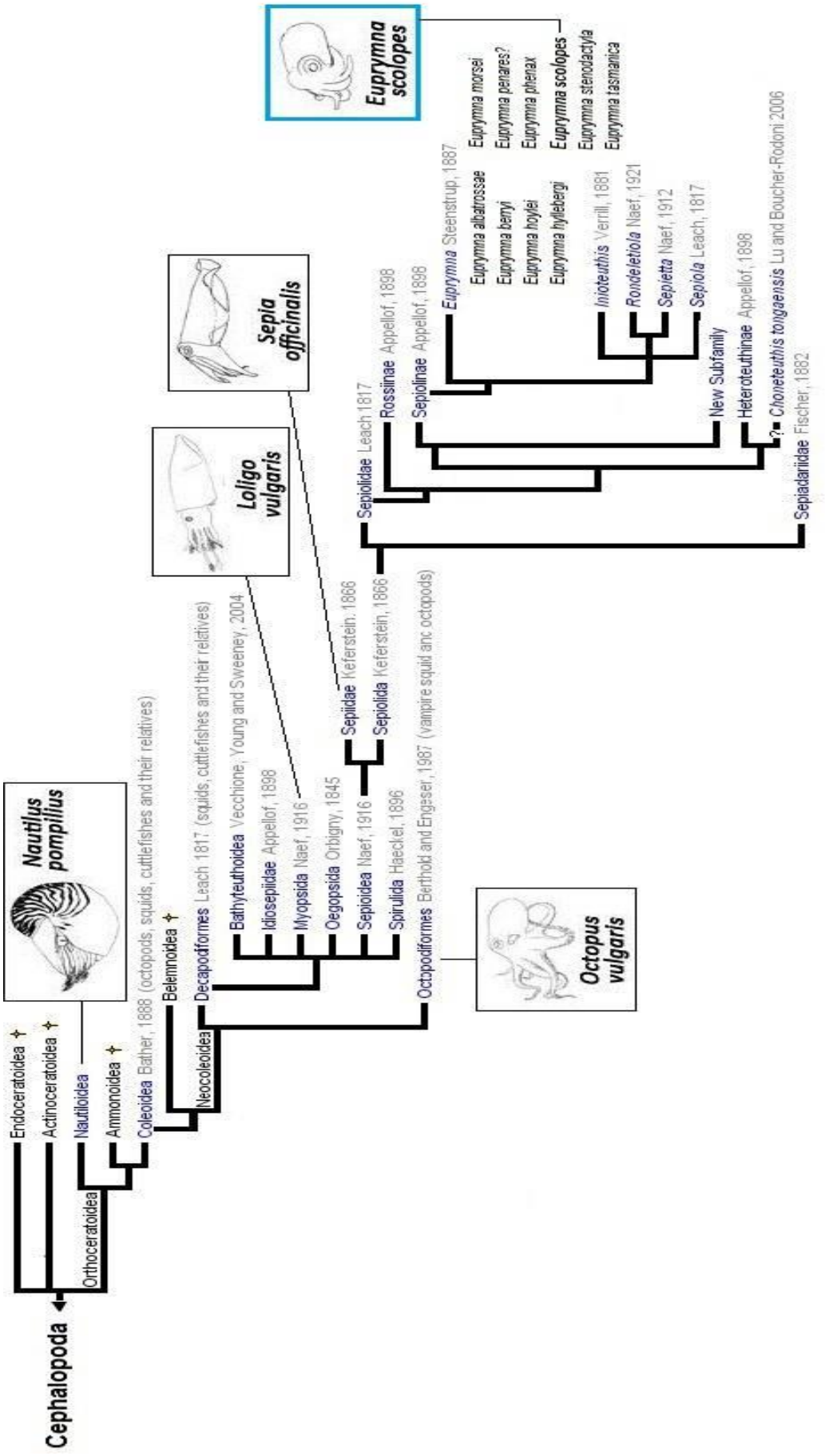


Fig. 1 Tree of Life: From Cephalopoda to the species Euprymna scolopes. Euprymna scolopes belongs to the Sepiolidae family.
Data taken from Tree of Life Web Project (http://tolweb.org) on December the 12th 2014 and have been put together manually.

Materials and Methods

For the description of the embryos and the adults I used the axes of the adult functional orientation rather than the embryo's morphological orientation. [**Fig. 2**]

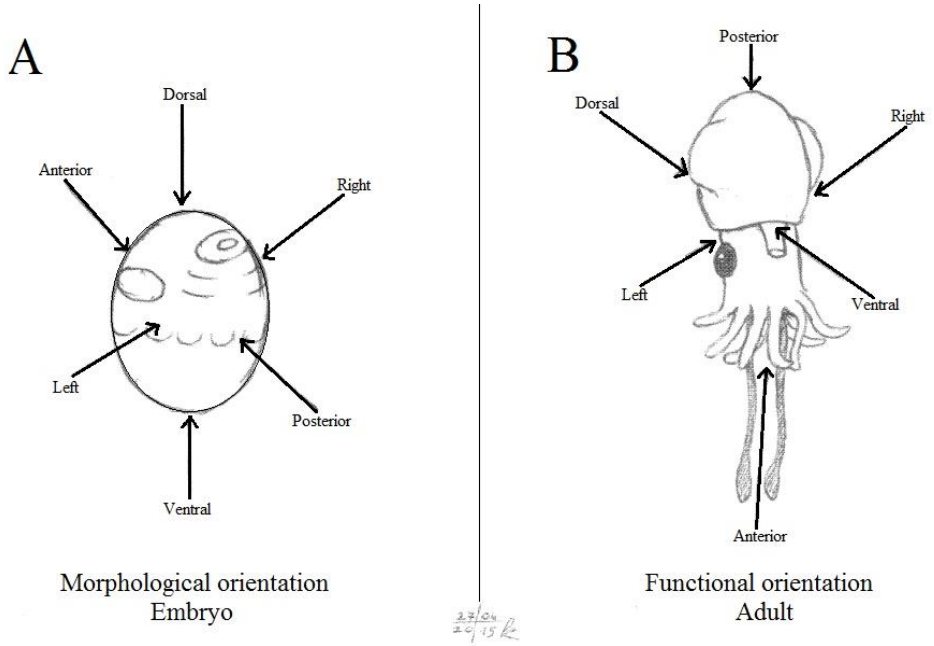


Fig. 2 Morphological and functional body axes in Cephalopods. (A)The morphological body axes of cephalopods are homologous to the body axes of other mollusks and are here shown on the left on the example of an embryo. In this orientation, the location of the embryonic mouth is anterior, the funnel is posterior, the mantle is dorsal, and the arms are ventral. (B) The functional axes of an adult cephalopod differ from the embryonic morphological ones. The location of the mouth is anterior, the opposite side of the body is posterior, the funnel is ventral, and the mantle is dorsal. Based on Lee et al. 2009b.

Specimens

Adults of *Euprymna scolopes* Berry, 1913 (Cephalopoda: Sepiolidae) were previously collected by Marie-Therese Nödl near the coast of Manoa, Hawaii. They were kept in through-flow aquaria at the University of Manoa, Hawaii, where they laid eggs. Those eggs were brooded at a water temperature of 24°C following the protocol of Lee et al. (2009b). Embryos of the needed stages 17-30 (where stage 30 is the hatching stage), determined after Arnold et al. (1972) and Lee et al. (2009a), were first manually separated from the jelly layer surrounding them, using tweezers. Then the embryos – still in their chorions – were prefixed for 1 hour in 4% paraformaldehyde in seawater. All embryos of stage 20 and above were anesthetized in 3.7% $MgCl_2$ in filtered seawater for 20 minutes prior to prefixing them. After the prefixation all embryos were washed several times in seawater. In all embryos older than stage 21 the chorion was removed manually. The animals used in this study were preserved in either 70% ethanol or 100% methanol and have been stored in the freezer at -80°C before being used in this study. [**Table 1**]

The adults were bred in the Monterey Bay Aquarium, California, euthanized there and sent to Austria with help of Chris Payne and Athena Copenhaver. For the transport they were stored in 10% buffered formalin solution. After arrival in Vienna we put them into 4F1G solution (4% formaldehyde + 1% glutaraldehyd in phosphate-buffered saline (PBS)) which minimizes the shrinking of the tissue. We received two female adults (called F1 and F2) and two male adults (called M1 and M2). The size varied between 2 cm and 3 cm and the males are slightly larger than the females.

Table 1 Embryos of different developmental stages and adults used in this study. Listed are the stages used, the type of fixation, staining and the number of animals stained and scanned. Adult F1 was restained and scanned a second time.

Stage	Day of development	Fixation	Staining	Number of animals stained/scanned
17	Not available			
18/19	Not available			
19/20	10-11	100% Methanol	PTA	4/4
21/22	Not available			
23/24	14-15	100% Methanol	PTA	3/2
25/26	16-17	100% Methanol	PTA	3/2
26	17	70% Ethanol	PTA	3/2
27	Not available			
28	19	70% Ethanol	PTA	3/2
29	20	70% Ethanol	PTA	2/2
30	21	70% Ethanol	PTA	3/2
Adult (F1)		4F1G	IKI	1/1
Adult (M1)		4F1G	IKI	1/1
Adult (F1)		100% Ethanol	I2E	1/1
Adult (M2)		100% Ethanol	I2E	1/1

Specimen preparation and MicroCT imaging

Embryos

As cephalopods belong to the mollusca, and therefore have no bony tissue, they do not naturally give a good X-ray microtomographic picture when being scanned. Therefore we tested two different X-ray-contrast staining methods for the embryos (Metscher 2009a): PTA (phosphotungstic acid) and IKI (iodine potassium iodine – one formulation of Lugol's solution). For a preliminary test three specimens have been stained with 1% PTA (phosphotungstic acid) in either ethanol or methanol according to the liquid in which they had been fixed. Three other specimens of equal stages have been stained with 2% KI + 1% elemental iodine (IKI). All six specimens stayed for about 25 hours in the staining solution and then the solution was replaced by distilled water to remove all the stain not taken up by the tissue. After scanning those test-individuals, we found that PTA is better to use on embryos of *E. scolopes* since IKI did not show the organs so well and the contrast was not as good as in the PTA-scans, making slightly blurrier pictures.

All embryonic individuals were stained in PTA following the protocol by Metscher 2009a. The stained animals had to be mounted in order to be scanned. For mounting I used a pipette-tip whose tip was sealed by melting it shortly over a flame. Then alcohol – either ethanol or methanol according to the used specimens – was injected to the bottom with a hypodermic needle. Next agarose in H_2O was melted in the microwave and was also injected into the pipette-tip. It is important to keep both, the agarose and the alcohol layer, free of bubbles. Now the embryo got positioned in the agarose layer, using fine-pointed tweezers (Federpinzette). All the steps from the injection of agarose onwards were repeated, after the first agarose layer was bonded, to position a second specimen of the same stage in the pipette-tip. The individuals have to be within a 35mm range from bottom to top, since this is the range the microCT scanner can move the probe up and down to get a perfect picture.

Pipette tips are good to use for scans of specimens stored in liquid because they have very thin walls (200-300 µm) and their conical shape allows the sample to rest stably with a minimum amount of medium surrounding it (Metscher 2009b). The pipette tips were placed in the microCT scanner on top of some Lego© pieces in order to put it in the optimum position for scanning. The tips are fixed with glue pads of UHU Patafix© to the topmost Lego© piece so they cannot move and stick firmly to the Lego©. [**Fig. 3**]

To take the microCT pictures of the embryos we used the Xradia MicroXCT scanner and the XMController MicroXCT 8.1.6599 program.

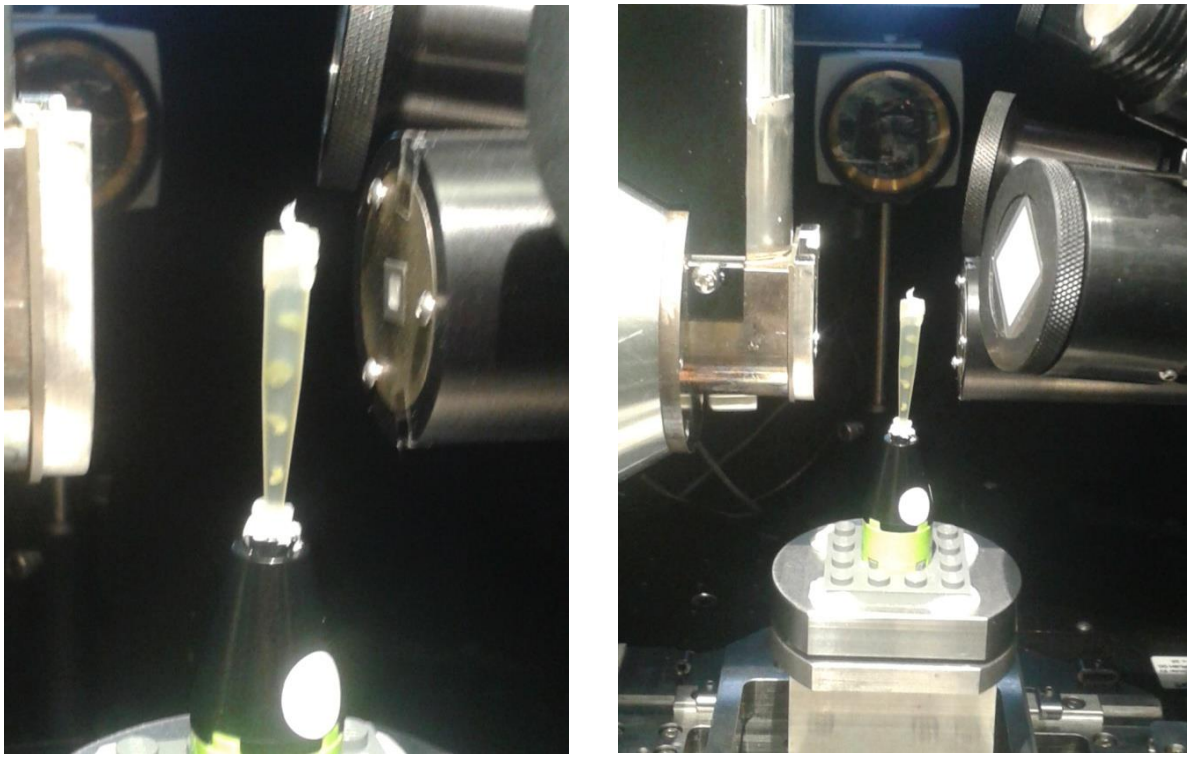


Fig. 3 Mounting of embryos in the microCT scanner. The embryos got fixated in agarose within a pipette tip which is sealed with a piece of Parafilm. For mounting it in the microCT scanner the tip is placed on top of Lego© pieces and fixed with glue pads.

Adults

Two of the adults (F1 and M1) were stained in 10% IKI (in distilled water) for 18 days and were check-scanned (just a short scan through the thickest part of the individual which takes only half an hour to see if the stain made its way all through the body) three times during this period to see if the staining had reached all of the animal. After this time the adults were changed to undiluted IKI for a more intense staining for another 3 days and after this they were finally scanned completely using a Sky Scan 1174 microCT system (www.skyscan.be). We chose IKI for the adults since PTA is a larger molecule and therefore penetrates slower and would probably not reach all of the tissue (Metscher 2009a).

The adults were mounted in a plastic tube with a piece of wax to keep them above the bottom and were fixed with two straws so they cannot move while scanning. Also a small amount of

distilled water was put in the tube below the wax in order to prevent drying-up of the specimens during scanning. [**Fig. 4**]

 f

Fig. 4 Mounting of adults. Left: Female 1 mounted for a check-scan within the Sky Scan 1174. Right: Male 1 Mounted for the total scan, fixed with two straws so it cannot move during scanning and having some distilled water to prevent drying out. The dark color of both is due to the iodine staining, which provides X-ray contrast to the tissues.

After the first scans of the adults F1 and M1 with the Sky Scan 1174 –which had to be used due to the size of the adults – we decided to stain F1 and M2 with another staining called I2E (1% iodine in absolute ethanol; Metscher 2009a). M1 unfortunately was a little distorted after mounting it for the first scan so we used M2. In order to use this staining on the animals we had to change their storage medium to 100% ethanol first. We did this by a step by step increase of alcohol concentration. Starting with 25% ethanol the concentration then was increased to 50%, 70%, 90% and finally to 100% ethanol. The animals were kept at least 3 hours in every alcoholic solution. After one night in 100% ethanol we placed F1 and M2 into undiluted I2E. Due to the alcohol used here the iodine is supposed to stick even better to the animal than in the usage of IKI.

For the second scan of the adults we used the Xradia MicroXCT-200 from the Department of Structural and Functional Botany at Rennweg 14, Vienna. As a controller program we used

MicroXCT 8.1.6599. This newer version of Xradia MicroXCT scanner owns a macro lens for bigger objects to scan – such as our adult *Euprymna scolopes*. For this scan F1 and M2 were mounted in tighter tubes using cut-in-half straws for preventing them from moving.

3D reconstruction

I used Amira 5.6.0 for reconstructing the *Euprymna scolopes* embryos and adults. I chose to use volume rendering for depicting the embryos and organs since it does not show the plasticine-like look that surface rendering shows. I started with the hatchling stage (stage 30) and created a color-map for every organ and saved it with the stage 30 reconstruction. I loaded the required colors from this map for every stage, so the organs have the same color throughout all the stages.

For marking the different organ structures I used the segmentation editor of Amira. I utilized the masking and the brush-tool for all the embryos and slices. For organs as large as the inner and outer yolk sac I also was able to use the interpolation tool. For smaller and more capillary organs, such as the gills and the heart, I could not use the interpolation because there were too many false structures marked.

For a first overview and for looking at plane cuts through the embryos I used Fiji (which is the same as ImageJ and an open-source program). Fiji facilitated the search for distinct structures and was great for giving a first inspection of the slices since the embryos could be cut in linear slices – whereas the segmentation editor of Amira always chooses a slightly diagonal way to cut the slices.

Dissection

In order to obtain a better impression of the organ morphology and location I chose to dissect Male 1 since it was no longer useful for scanning due to the deformation caused by the first mounting. I dissected it under a dissecting microscope using needles to pin it to the ground in order to avoid movement during dissection, and tweezers and a scalpel.

I used the *Loligo sp.* dissecting manual of the anatomical dissecting book “Kükenthal – Zoologisches Praktikum” by Storch and Welsch (Storch & Welsch 2009) as a basic template, although there were some differences – of course – to *Euprymna scolopes*.

Construction of the online 3D Atlas

The overview of the 3D atlas is categorized into the different developmental stages and the adults with a link to the original stack, the marked and labeled images and the metadata for each specimen. The labeling of the reconstructed three dimensional pictures was done by using Microsoft Paint.

To publish the resulting data I chose the online platform morphdbase.de (MDB). On the online platform the arrangement of the published data differs from the 3D atlas overview. There the first information the user gets are the marked and labeled images of the different stages including a link to the original microCT stacks which are uploaded on the same platform. In the case of the adults the data of the adult male and female as well as the close-up scan of the male buccal mass, are combined in one entry including not only the link to all of the three original stacks but also to the rotating video of the adult male.

The project of developing and installing the MorphDBase webpage was funded by the German Research Foundation (DFG). The MDB platform was installed in 2006 with the goal to establish a platform for morphological data similar to the already existing GenBank platform for genetic information. Morph D Base is not only for storing and documenting morphological data but also aids communication and collaboration by sharing information and discussing structures and their possible interpretations. Therefore it also allows descriptions and comments on the datasets. Furthermore, in order to enable proper documentation and proof for the descriptions, it is possible to upload media files to the data base as well. It also contains a taxonomy browser and can be used by various internet browser programs. Since the online 3D atlas of *Euprymna scolopes* contains only microCT stacks in TIFF format, pictures in JPEG format and one movie in MPG format all of those are placed in the category media.

Results

Dissection

I dissected Male 1 and made sketches which I also used as a help for marking the organs in Amira. [**Fig. 5**, **Fig. 6**, **Fig. 7**]

I used the chapter of a *Loligo sp.* dissection of the anatomical dissecting book "Kükenthal – Zoologisches Praktikum" by Storch and Welsch (Storch & Welsch 2009) as a guide for the dissection of Male 1. Within the book there was also a guide to dissection *Sepia officinalis* but since *S. officinalis* has a shell inside and *Loligo sp.* and *E. scolopes* do not, I decided on using *Loligo sp.* as a guide.

The dissection started with a ventral cut of the mantle in order to show the full size of the funnel as well as the organ sac containing most organs. The gills and gill hearts are located outside of the organ sac since they have to get in contact with the water which enters the mantle cavity through the funnel. The gills were connected to the mantle-tissue. Next the funnel was removed and after removing it, it was visible that the light organ as well as the rectum and the ink sac were located outside of the organ sac too. Then the organ sac was opened in order to see all of the organs visible from the ventral side. [**Fig. 5**]

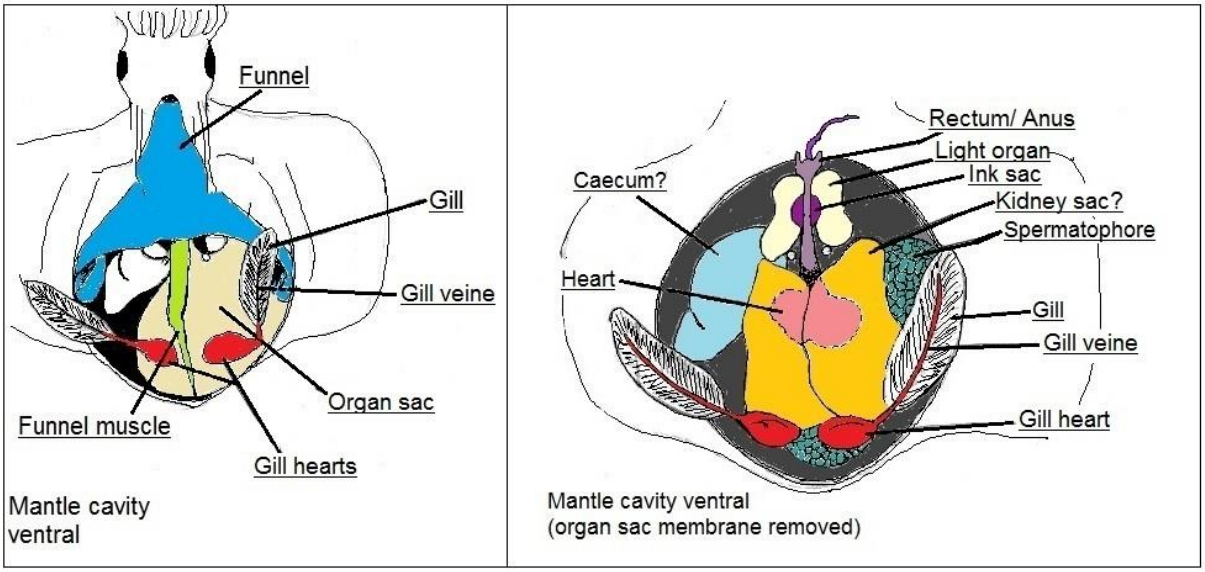


Fig. 5 Sketches of dissection of Male 1 (1). Left: mantle cavity opened from ventral side. Right: mantle cavity with opened organ sac. The big sac marked with "Caecum?" might be a digestive gland but could not be determined exactly. Same for the "Kidney sac?".

After taking a careful look at the organs within the body I detached the organs from the mantle cavity and removed the whole digestive tract and adhesive organs in order to get a better overview of the connections within the body. It showed that the digestive tract is U-shaped and that most organs structures are connected. [**Fig. 6**]

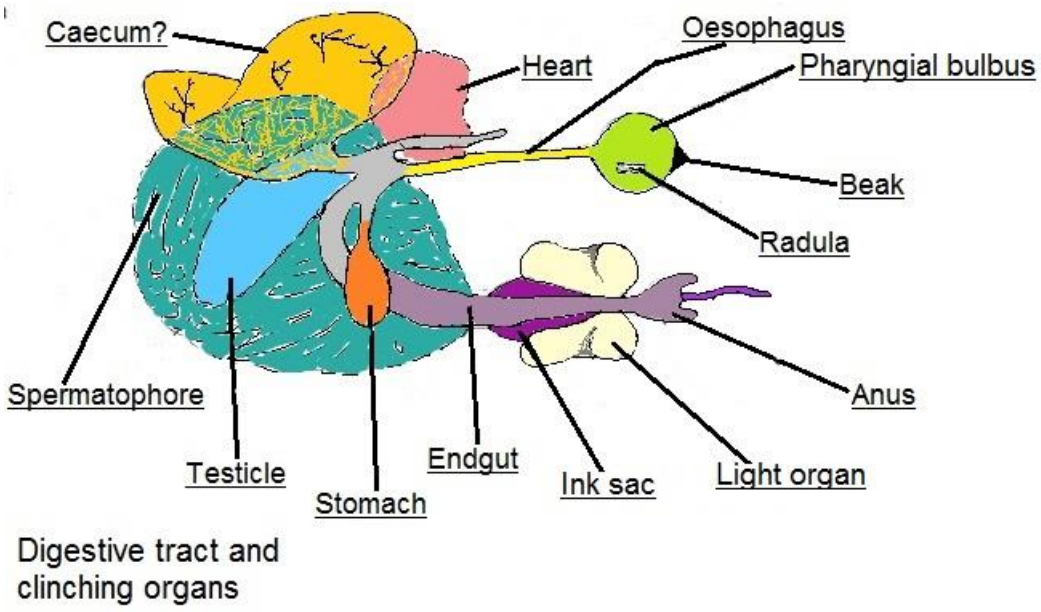


Fig. 6 Sketch of dissection of Male 1 (2): the digestive tract and the clinching organs outside of the body cavity.

In the end of the dissection I took a closer look at the beak and its different components and also at the structures of the funnel. [**Fig. 7**]

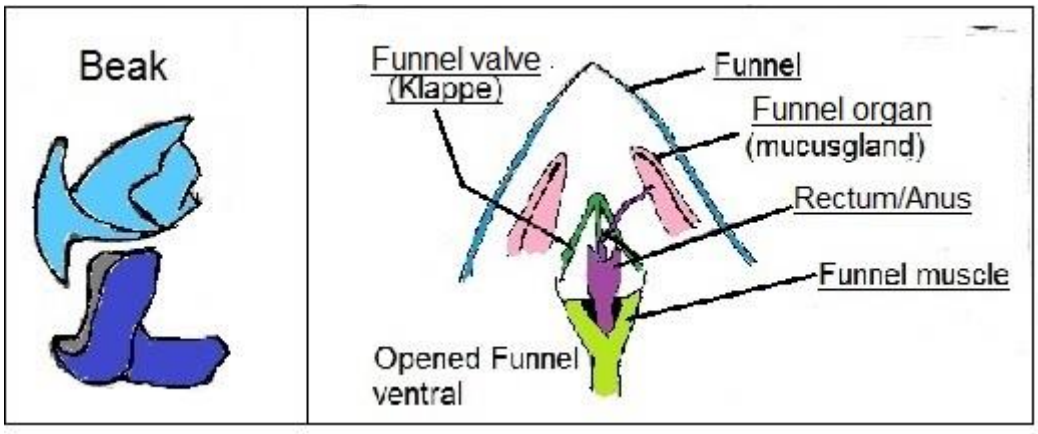


Fig. 7 Sketch of dissection of Male 1 – details. Left: Beak top (consisting of three pieces) and bottom (consisting of two pieces). Right: ventrally opened funnel, the tip pointing anterior.

There were some inconsistencies concerning the organs I found and the organs described in the book, which is probably due to the fact that the guide shows another species of cephalopod. The organ marked as “Caecum?” in the sketches could be a digestive gland in *Euprymna scolopes*. Such a gland is described in *Sepia officinalis*, but is located there on the very ventral side while in *E. scolopes* it is located on the very dorsal side. The organ marked as “Kidney sac?” is difficult to interpret since I could not find it in the scans at all. In *Loligo sp.* such a kidney sac is located at the ventral side, but I am uncertain if it is the same structure in *Eurprymna scolopes* because of it not being visible in the scans.

Adult organs

The following descriptions start with the adult stages and progress towards the earlier embryonic stages, since the adults give a complete overview of the organ topography when development is finished. A first overview of all the stages scanned is useful to familiarize oneself with the appearance of the animals. [**Fehler! Verweisquelle konnte nicht gefunden erden.**]

In order to provide a complete description of the adult *Euprymna scolopes* a male and female adult were scanned. Since most organs were easier to identify in the scanned male all of the labeled pictures – except for the ones showing the female reproductive system – are depicting the male individual (M2). Also the close-up scan of the buccal mass shows the buccal mass of the male adult.

The adults were delivered to me after I already finished labeling the embryonic stages. Therefore I labeled the adults using the knowledge I had gained from the scans of the embryos and comparing them to what I found in anatomical drawings of *Loligo sp.* . The dissection of Male 1 and the fact that I saw the organs in their fully grown form induced me to make some corrections in the labeling of the embryonic stages.

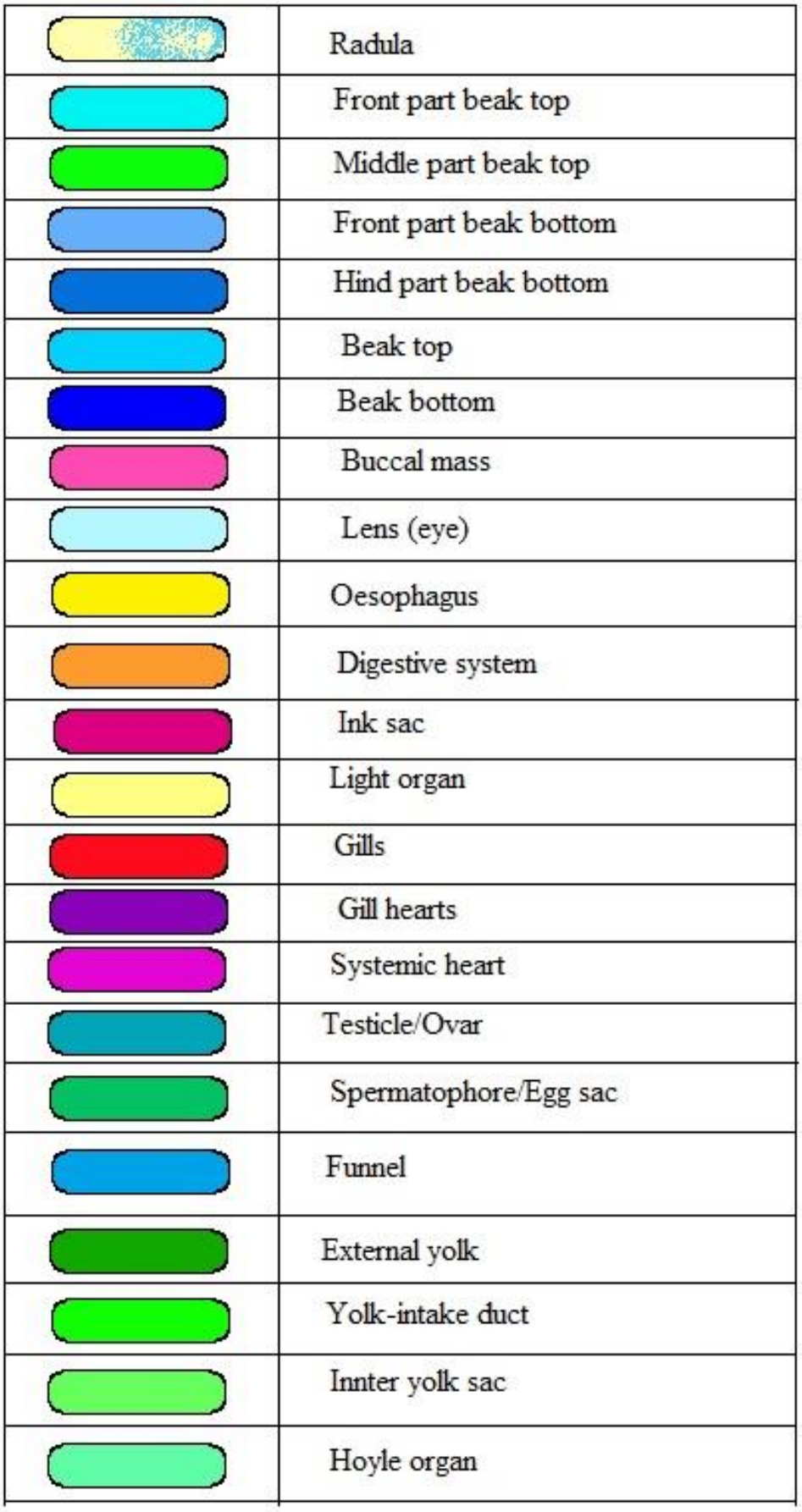


Fig. 8 Colortable. Colortable for both embryonic and adult scans showing the color indications of the different organs.Not all organs listed here are found in all scans. The different beak parts only concern the buccal mass close-up scan and the last four only concern the embryonic stages.

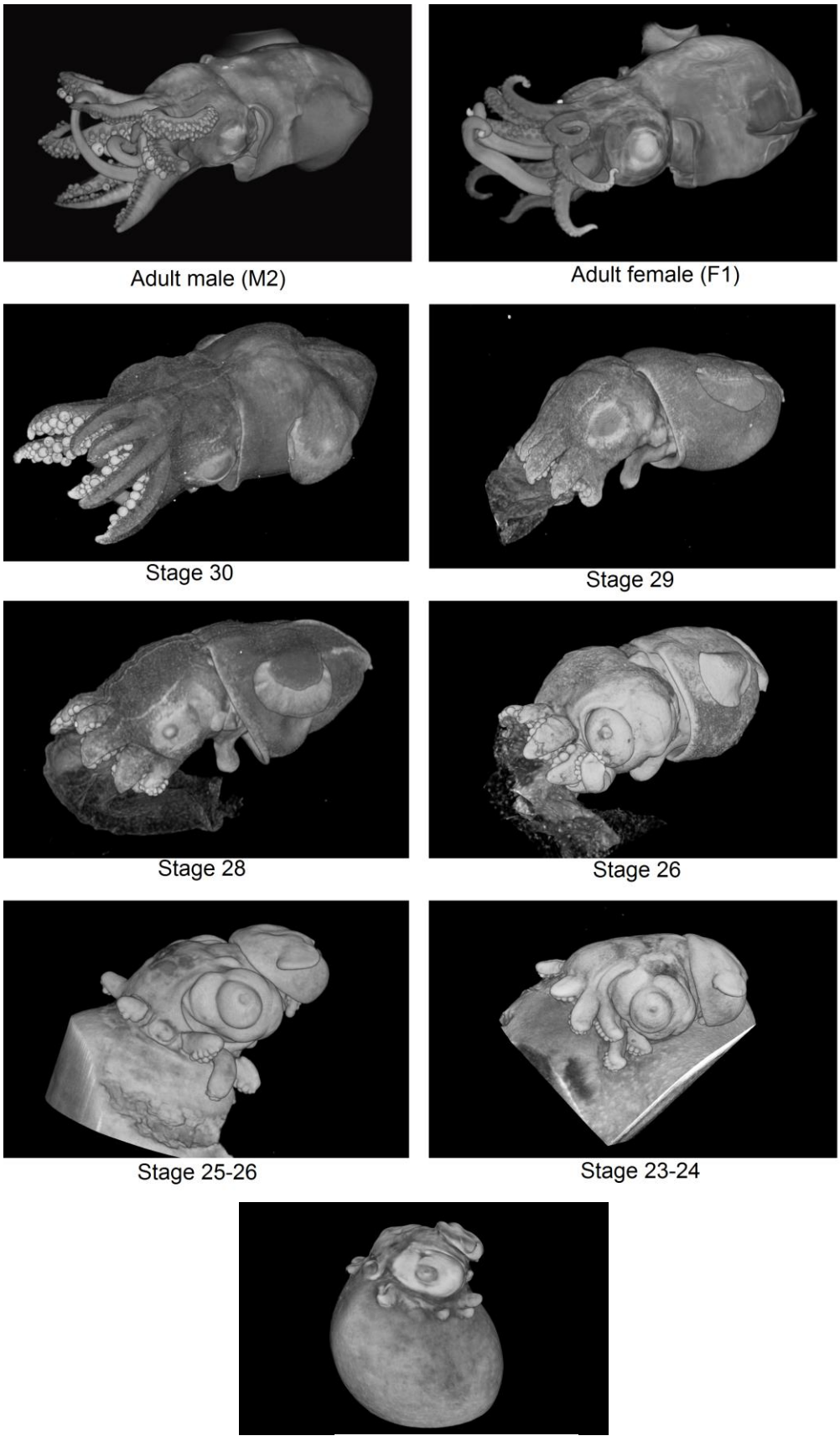
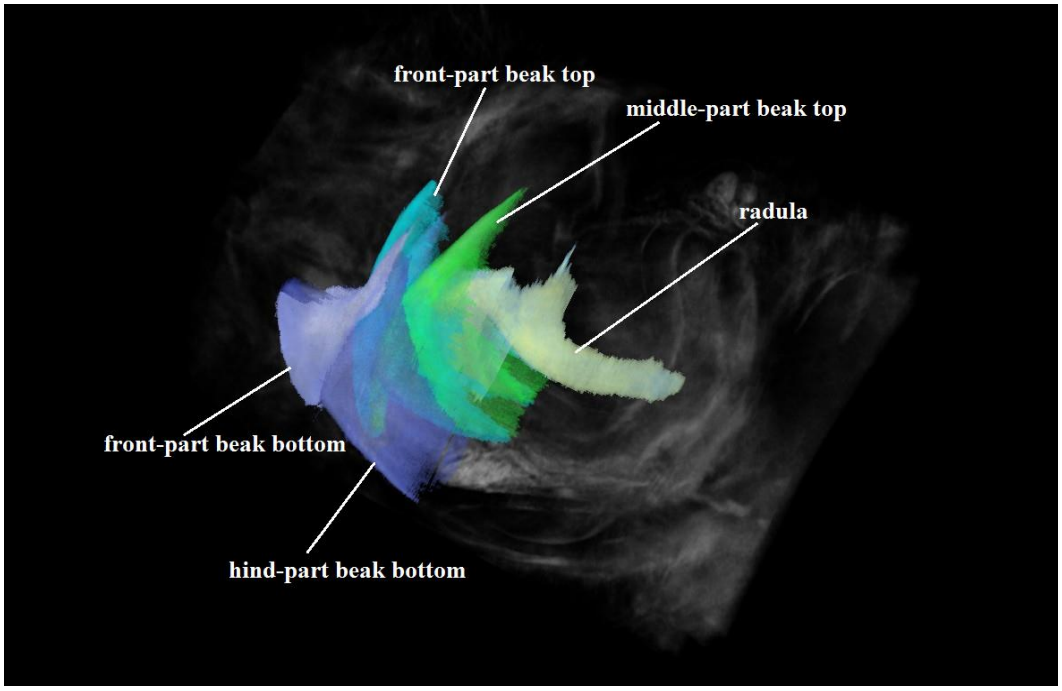


Fig. 9 Overview of the scanned individuals. Left to right and top to bottom

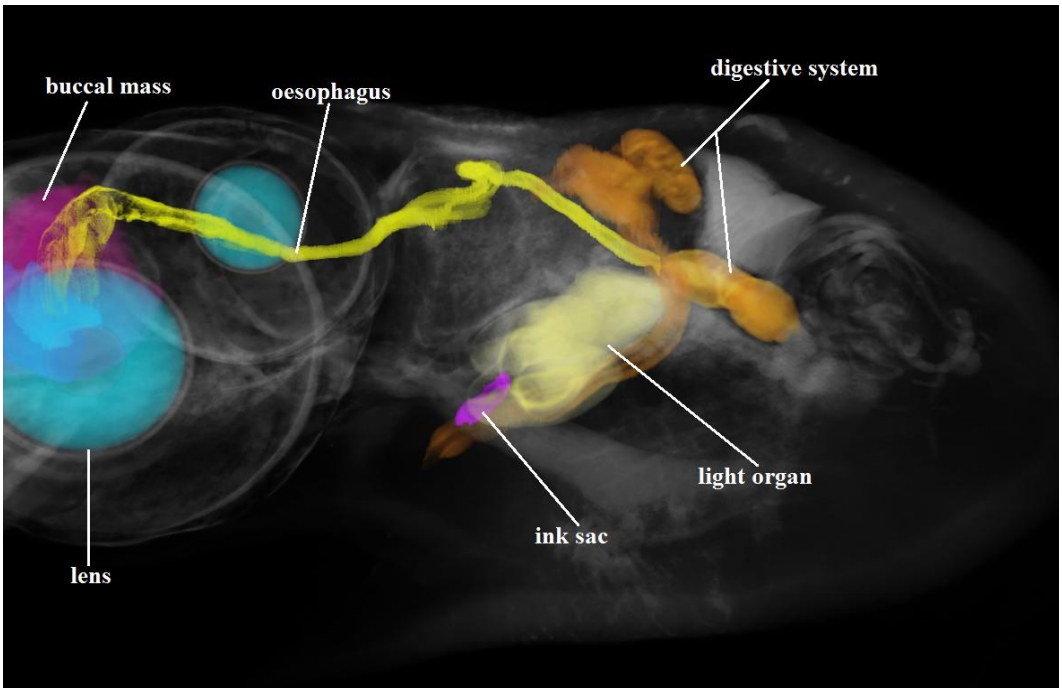
Adult Male (M2)

Total body length: 33.6 mm

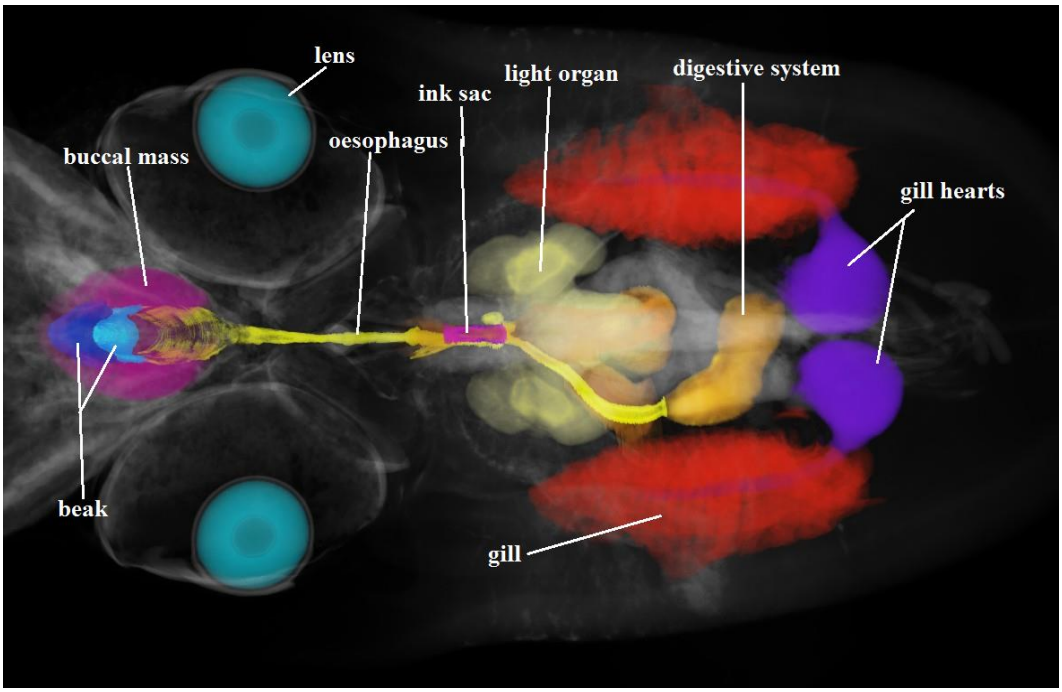
The organs were very well visible and I will start the description from anterior to posterior and from inside to outside.



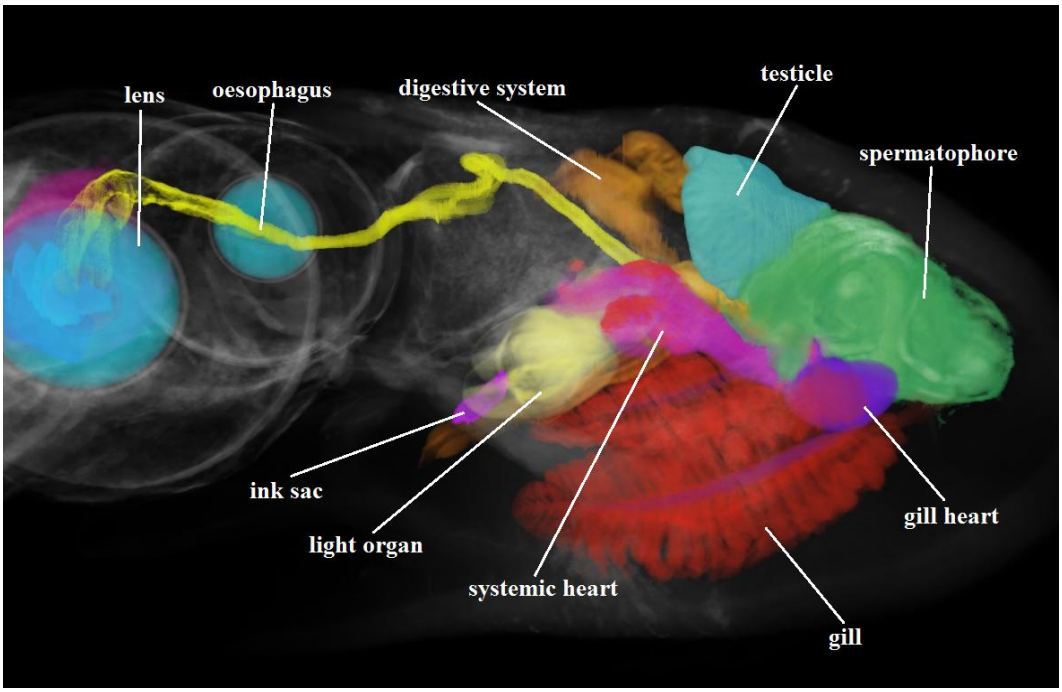
Picture 1: Buccal mass close-up scan lateral view. The radula was visible after a close-up scan of the buccal region. Without microCT it would not have been able to find it at all. It is U-shaped and lies side-wards with the open end facing posterior. The chitinuous teeth arise at the dorsal side and border the U-turn of the radula to its ventral side. In the close-up scan also most parts of the beak are visible. The top-half of the beak consists of 3 pieces whereof two are visible in this scan. The bottom-half of the beak consists of 2 pieces which are both visible here. To get an idea of the beak-pieces take a look at the dissecting sketch. [**Fig. 7**]



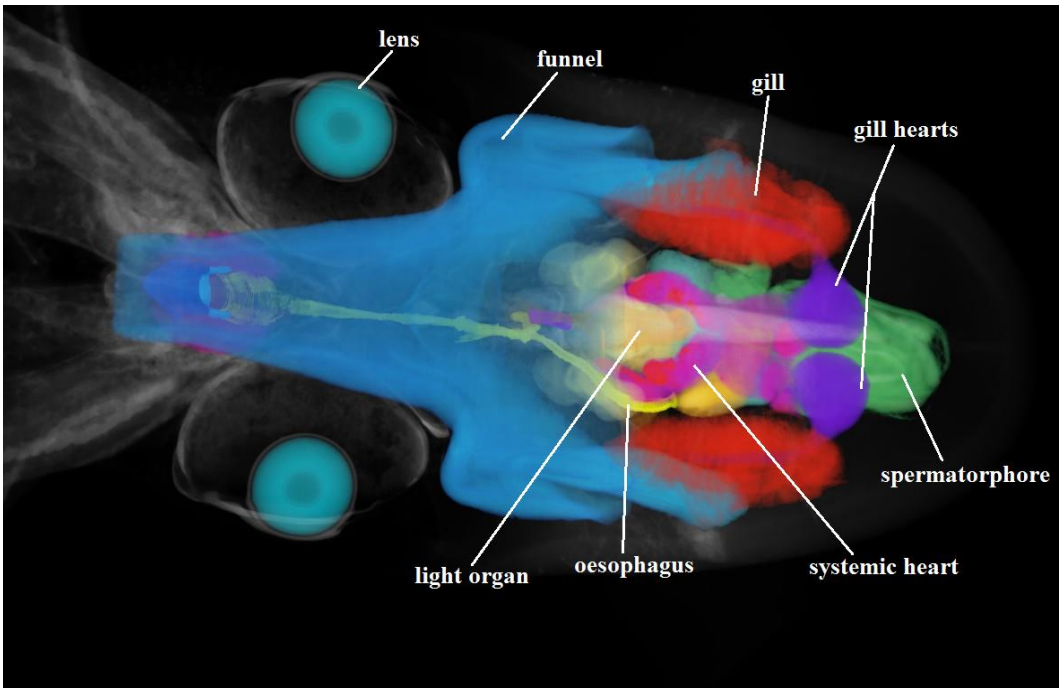
Picture 2: Lateral view. The buccal mass surrounds the mouth cavity and therefore also the beak and radula. Following the buccal mass the oesophagus leads from the mouth to the digestive system. The digestive system in its totality is also U-shaped but having the open end orientated to the anterior end of the body. Representing an anal-gland of the digestive system the ink sac is located at the very end of the gut, so the ink is – just as digestive products are – released through the anus into the mantle cavity from where it is ejected through the funnel into the open water. The light organ is located dorsally of the rectum.



Picture 3: Ventral view. When looking at the squid ventrally the double-kidney-shape of the light organ is visible. Also the large, feather shaped gills are apparent. They are located in the mantle cavity but outside of the organ sac. The round knobs at the base of the gills are the gill hearts which support the blood flow from the gills to the systemic heart and the rest of the body.



Picture 4: Lateral view. The gill hearts are connected to the systemic heart by large blood vessels. The systemic heart in the adult is a flattened tube, laying in the center of the body on the anterior-posterior axis. There are no blood vessels marked since they are no parenchymal organs. The next organs to look at belong to the reproductive system. In the male those are the testicle and spermatophore – both unpaired – located at the very posterior end of the organ sac and body. Due to restrictions of resolution the spermatic duct is not marked.

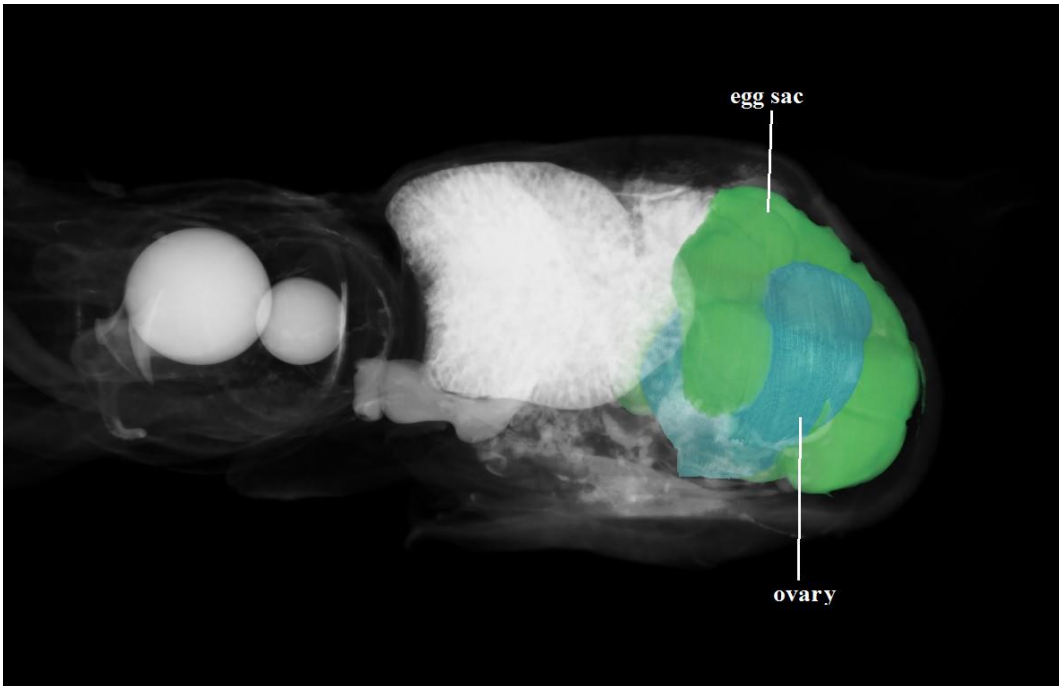


Picture 5: Ventral view. The last organ to describe in the adult is the funnel. The funnel covers nearly the whole ventral body side. It connects the mantle cavity with the surrounding water and therefore is responsible for the gills to receive enough fresh water. It also ejects the digestive products and ink into the surrounding water and is necessary for a fast flight of the animal. For the flight the water inside the mantle cavity is ejected very fast with high pressure by a contraction of the mantle muscles. The mantle muscles are also important for the direction of flight. Those muscles are not labeled here.

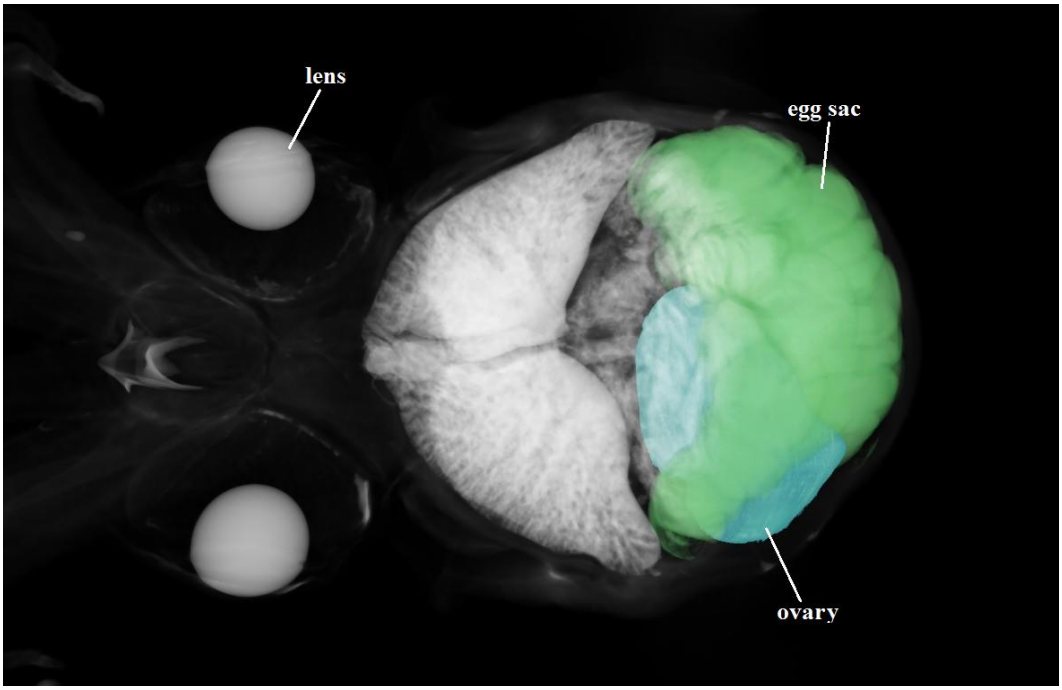
Adult Female (F1)

Total body length: 26.6 mm

Since the anatomy of the female is – except for the reproductive system – similar to the one of the male only the reproductive system is marked and labeled in these scans.



Picture 1: Lateral view. The ovary is located slightly more ventrally than the testicle in the male and the egg sac surrounds it partly. The bright white structure – that is even brighter here than it was in the male scan before – which is seated between the well visible eye lenses and the colored reproductive system, is the possible digestive gland adduced in the sketches of the dissection as “Caecum?”.



Picture 2: Dorsal view. A dorsal view shows that the egg sac in the female is much larger than the comparable spermatophore in the male is.

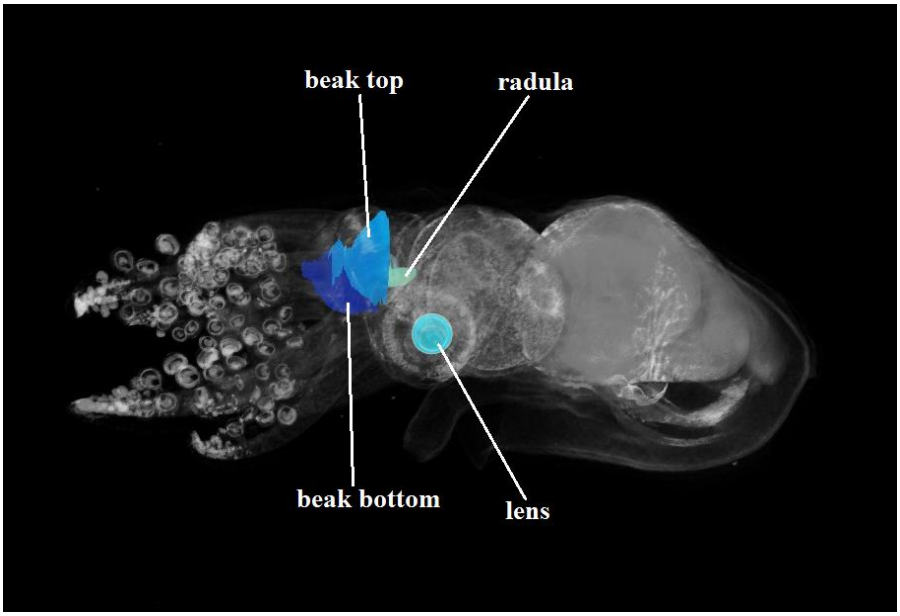
Developmental stage series

To get a good overview of the organs present in the embryos I first took a look at the slices of stage 30 in Fiji. Then I started marking the organs of stage 30 in Amira, proceeding towards the younger stages of development ending at stage 19/20. Previous to stage 19/20 only the yolk and the outer body layers are visible in microCT scans, so I only briefly monitored stage 17 to check whether there is also only yolk and outer body layers visible at that stage, which it was. For the stages between stage 30 and stage 19/20 detailed descriptions of the organs and organogenesis were performed. I took the eye lenses as an orientation point when orienting the whole animal and observing the marked organs only (by masking the outer body layers), which is why I marked the lenses too. The stages were determined according to the staging series of Lee et al. 2009b. For a better understanding of the time intervals of the transition from one stage to the next, the days after fertilization are also stated at the heading.

Stage 30 – Day 21 after fertilization

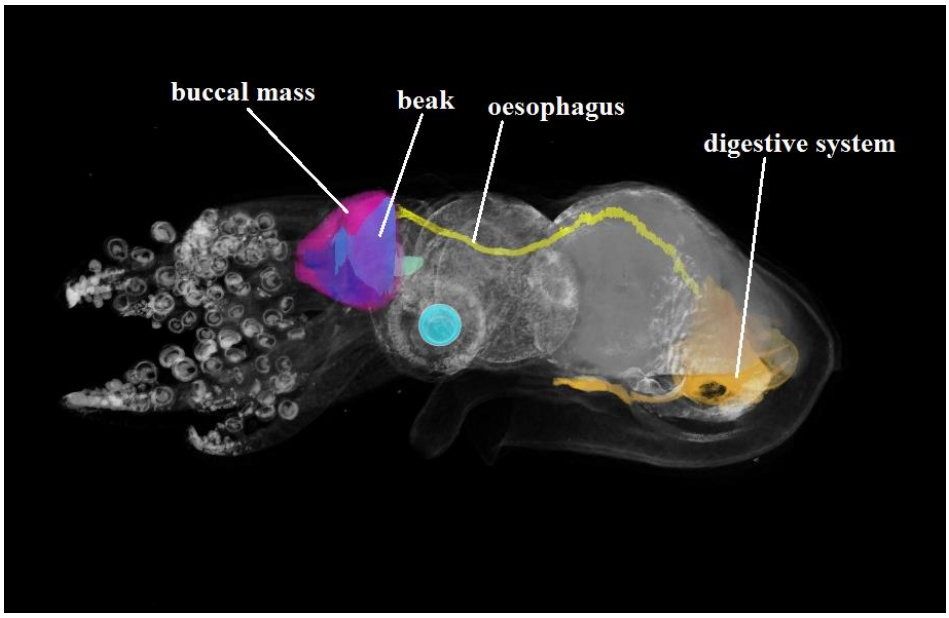
Total body length: 3.6 mm

The hatchling stage represents the final embryonic stage and therefore provides a good reference point for detecting all the organs and for knowing where to find the same organs in the earlier stages.

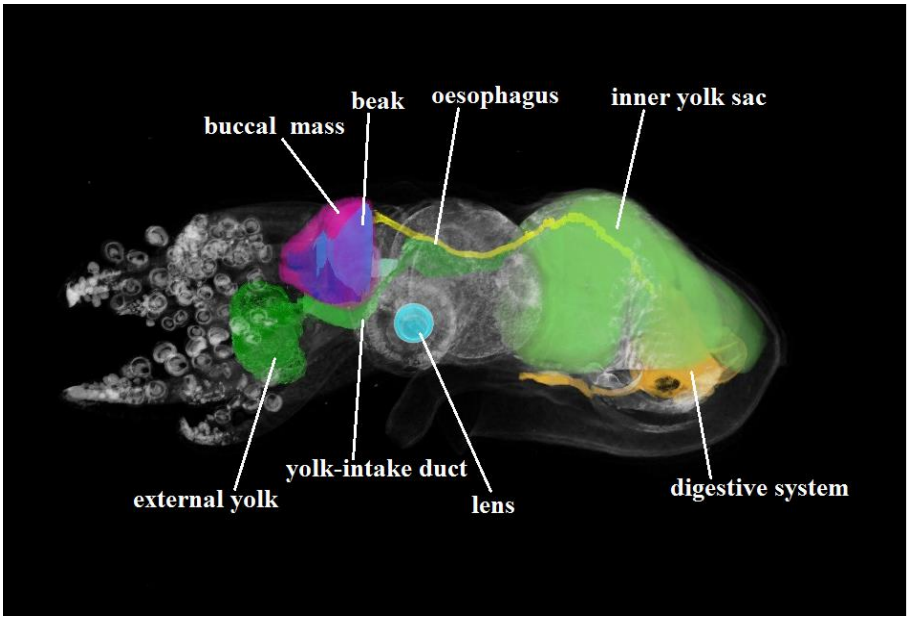


Picture 1: Lateral view. Most organs are easily visible. The two sides of the beak were hard to identify – especially to detect where one side ends and the other begins – whereas the radula

was very easy to find, since it literally lit up due to its chitin lamella which take up stain while still developing.

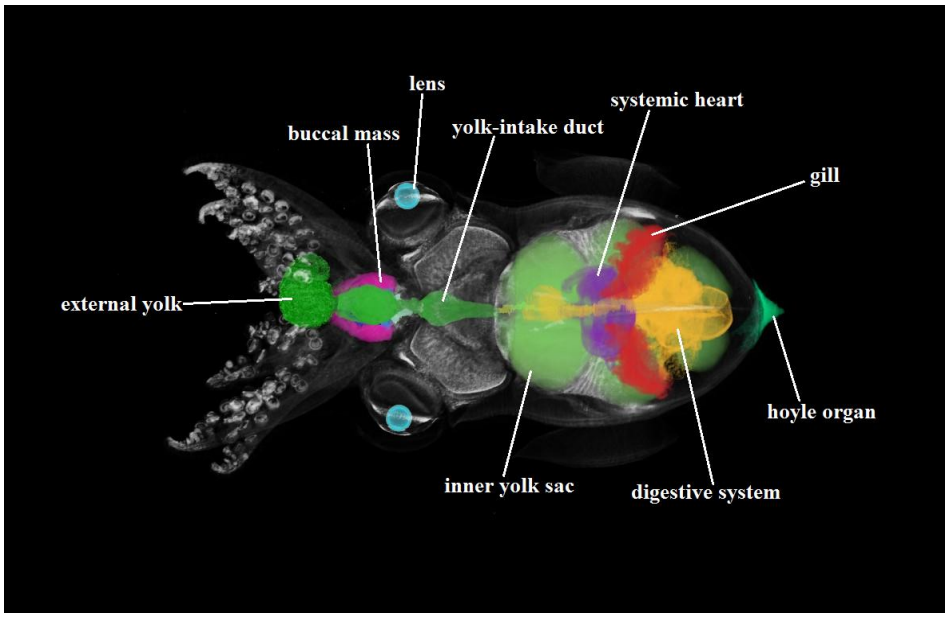


Picture 2: Lateral view. The buccal mass surrounds the beak and radula and is followed by the oesophagus which leads to the digestive system. The digestive system is not as distinct as in the adults.

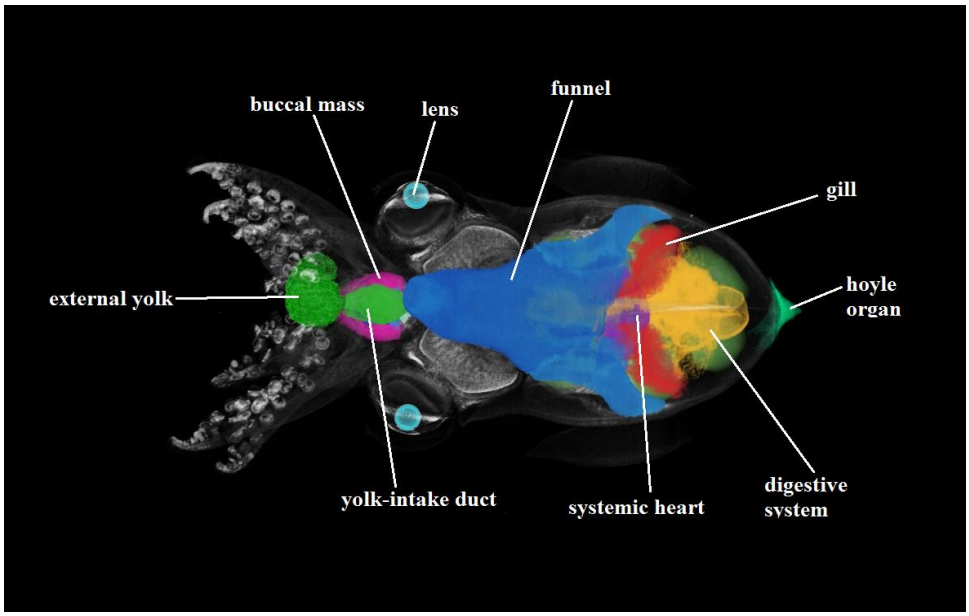


Picture 3: Lateral view. My first assumption was that the yolk is taken in through the oesophagus but short time later it became evident that there is a separate pathway for the

intake of yolk. The external yolk is connected to the already absorbed inner yolk in the inner yolk sac by the yolk-intake duct, as I called this newly found structure. The yolk system is entirely separate from the digestive system. Most likely the yolk-intake duct is closed during hatching and after hatching the young squid can live the first few days on the yolk reserve of the inner yolk sac. The inner yolk sac has four lobes at stage 30 and takes up most of the space of the body cavity.



Picture 4: Ventral view. From a ventral perspective the feather shaped gills and the systemic heart are visible. The systemic heart in the embryo is differently shaped than in the adults. It is not as elongated but still located in the center of the body. There are no gill hearts viewable. The yolk-intake duct is very well visible from this ventral point of view. At the very posterior end of the body the organ of Hoyle is located. It is a hardened spike which is used in the hatching process to penetrate the egg-membrane and enable the embryo to hatch.



Picture 5: Ventral view. Covering up nearly the whole ventral side of the embryo, is the funnel. It has reached its full size in relation to the body size and will only grow allometrically with the rest of the body to adult size.

The lateral dissection image made with Fiji provides a good overview of the organs. [**Fig. 10**]

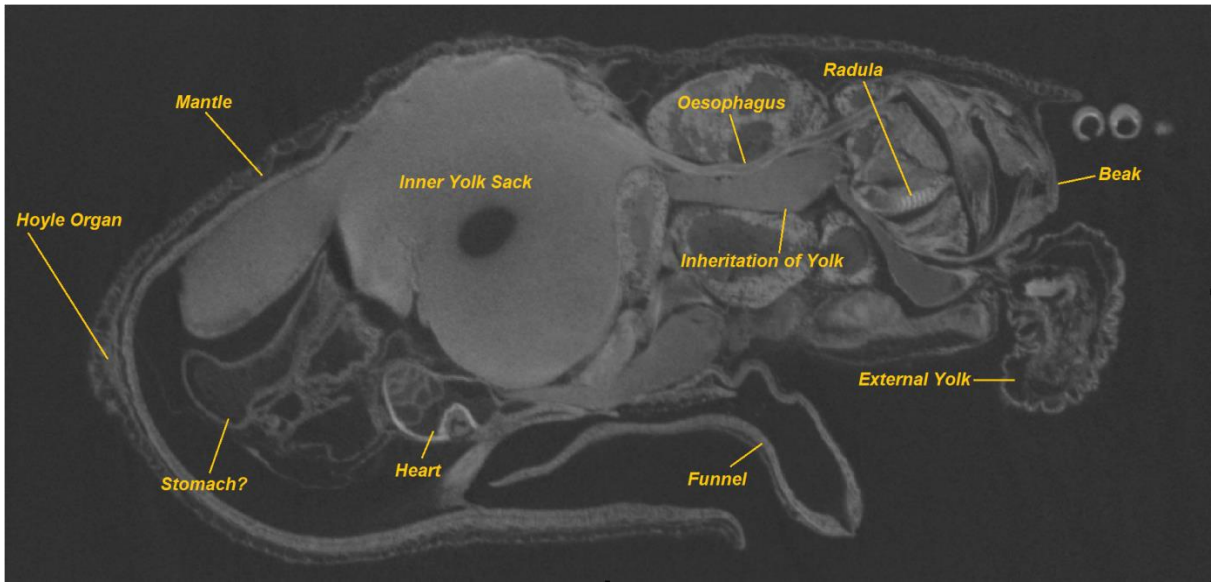
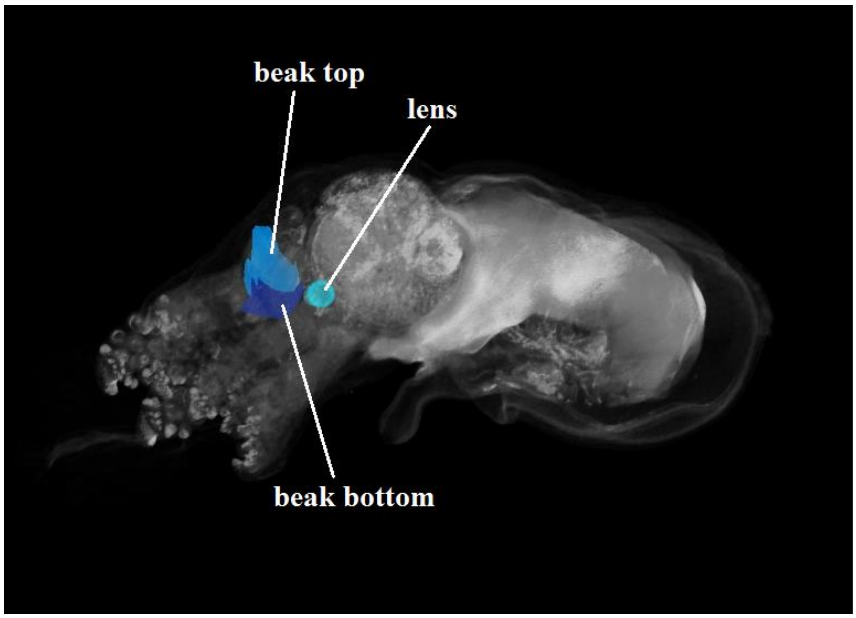


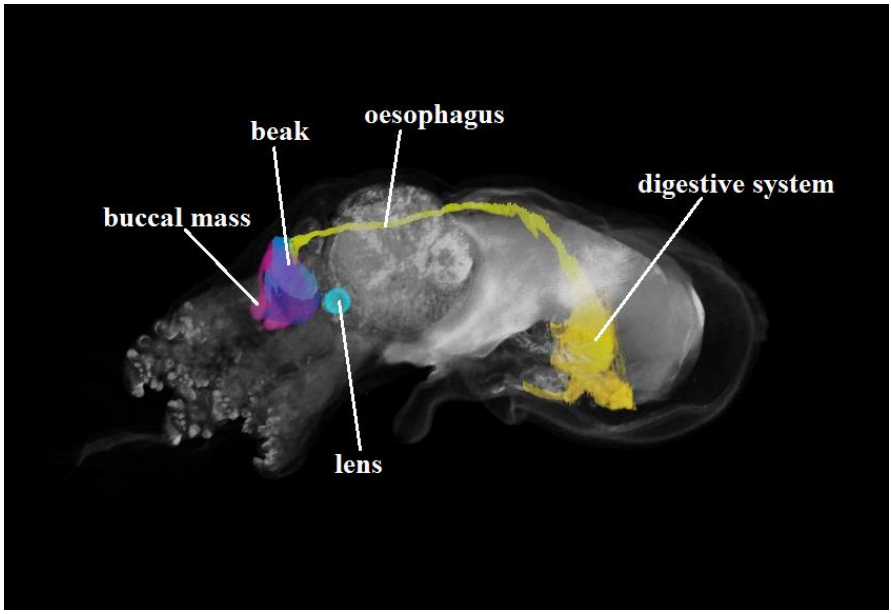
Fig. 10 Stage 30 longitudinal section in ImageJ showing the different pathways of the yolk-intake duct and the oesophagus as well as the radula, the beak, the external yolk, the funnel, the heart and a sac beyond the inner yolk sac which may belong to the digestive system and could be the stomach. Due to the other surrounding structures also visible in the same region, it is not certain that the marked sac-like structure is the stomach.

Stage 29 – Day 20 after fertilization

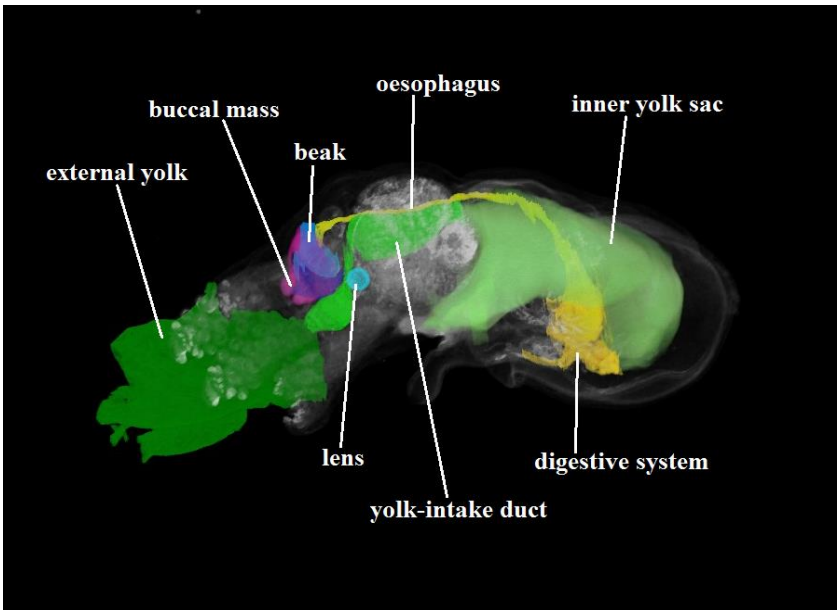
Total body length: 2.8 mm



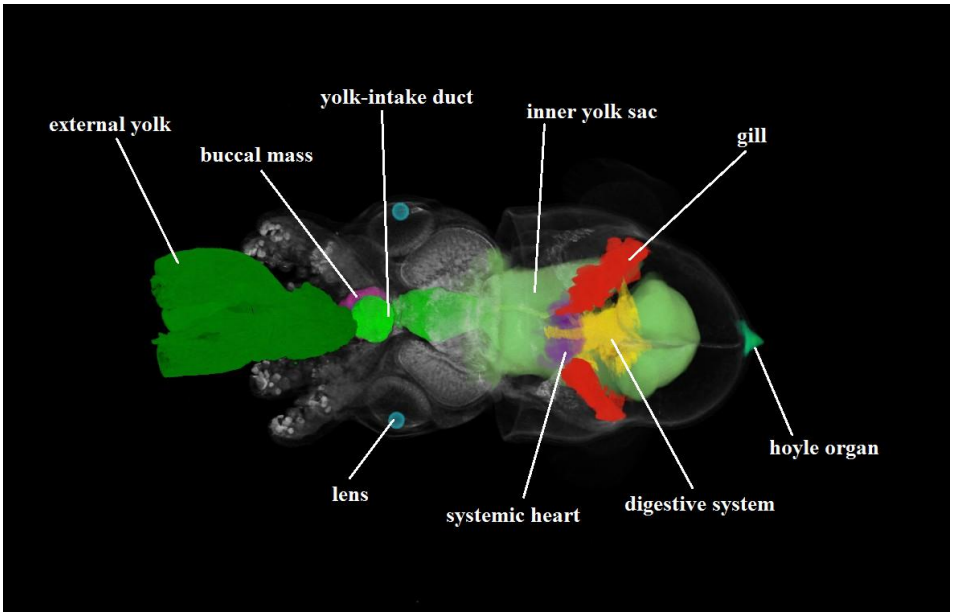
Picture 1: Lateral view. The top and bottom parts of the beak are well visible in stage 29 but already tend to look disproportionally large compared to the rest of the body. The radula is not apparitional. This indicates that the chitinous parts arise very late in development, shortly before they are needed.



Picture 2: Lateral view. The buccal mass surrounds the beak and the oesophagus connects the mouth cavity with the digestive system.

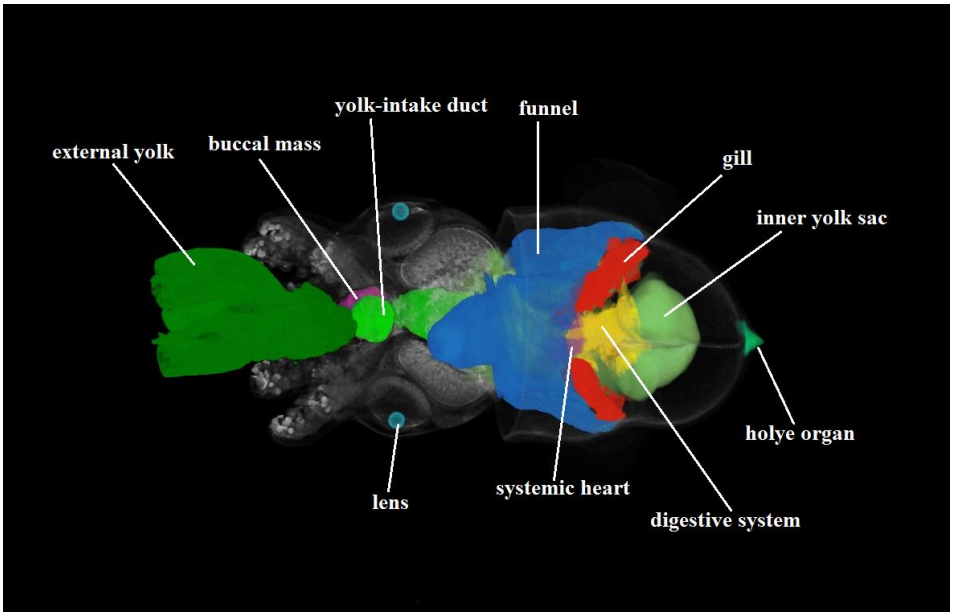


Picture 3: Lateral view. The external yolk is about the size of the head of the embryo. Again the inner yolk sac is connected to the external yolk by the yolk-intake duct. This duct is structured into two connected sacs where the first one is in direct contact with the external yolk and the second one is connected to the inner yolk sac by another small channel. The inner yolk sac has four lobes but does not take up all of the room in the body cavity.



Picture 4: Ventral view. The gills are much smaller and lost their feather-like shape and partly also the feather-like structure, which indicates that the gills grow in a last burst of growth shortly before hatching. The systemic heart is located in the same place as in stage 30 and it is

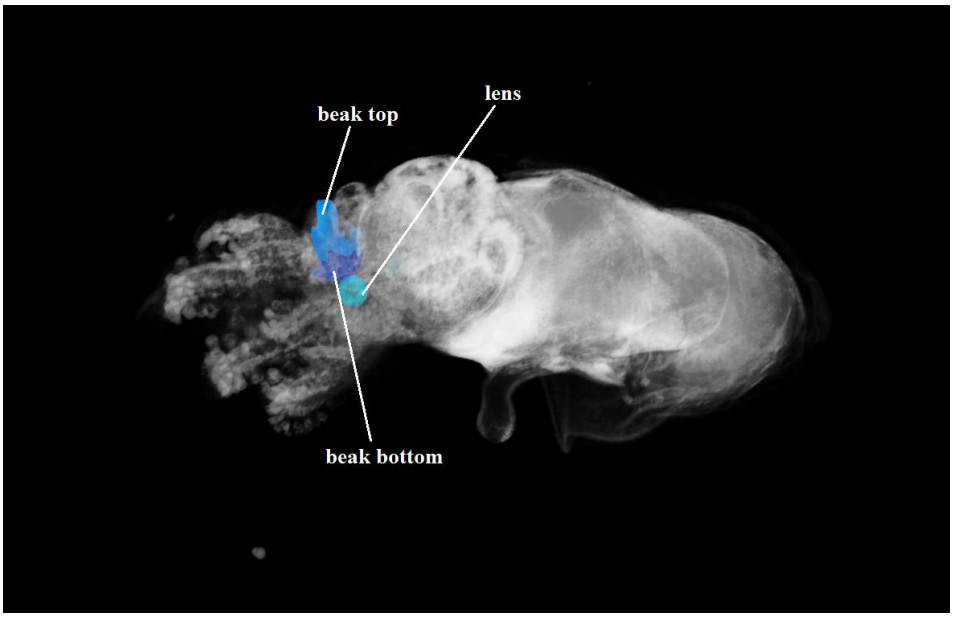
at almost the same size. At the very posterior end the Hoyle organ is visible, it is formed like a small spike.



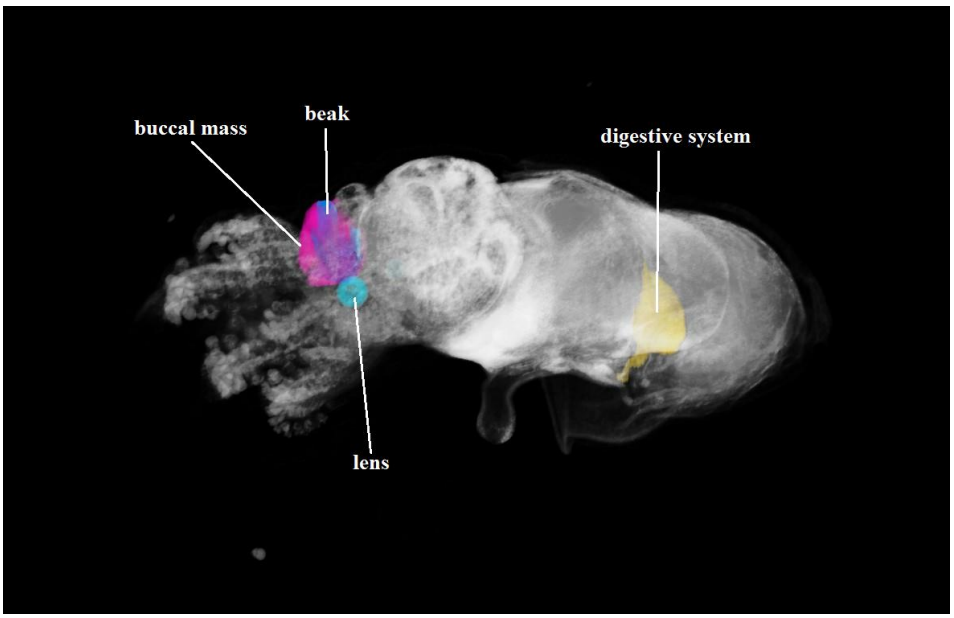
Picture 5:Ventral view. The funnel is visibly smaller and does only cover half of the ventral body side. Also the funnel ending is slightly bent. This bending of the funnel probably happened due to tissue shrinking during preservation or is a result of the small space within the egg and therefore of natural origin.

Stage 28 – Day 19 after fertilization

Total body length: 1.8 mm

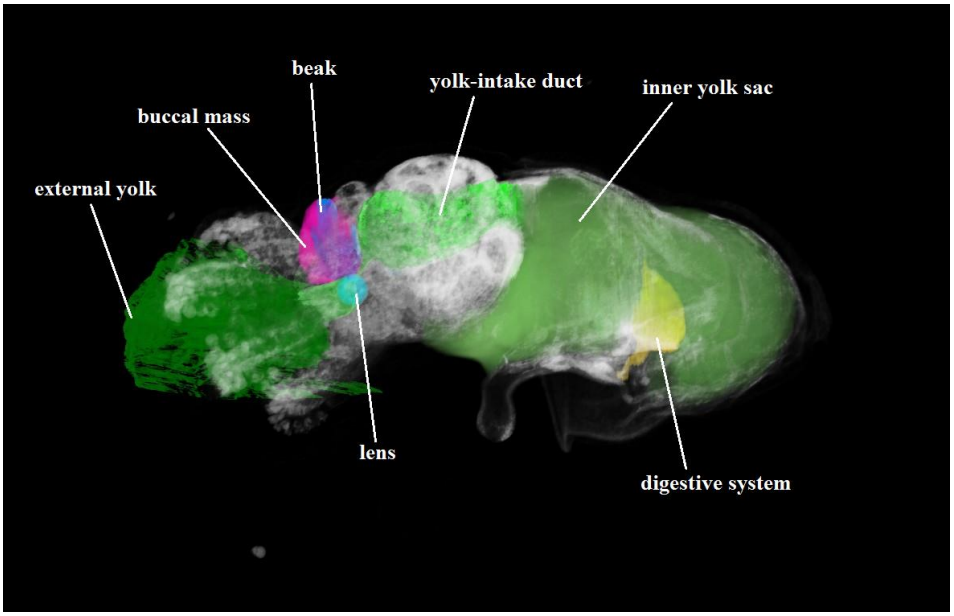


Picture 1: Lateral view. The body is about the same size as the head. The beak is still well visible even though it is smaller. Particularly the bottom part of the beak is visible as well as the top part.

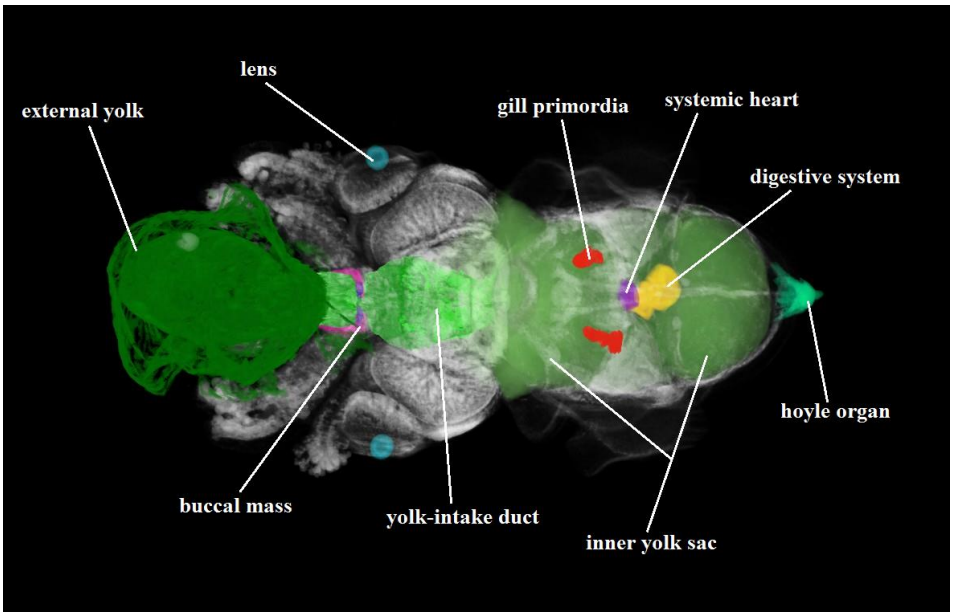


Picture 2: Lateral view. The buccal mass is not that thick and surrounds the small beak. It is not elongated towards the body. Due to resolution limits and the long preservation time the

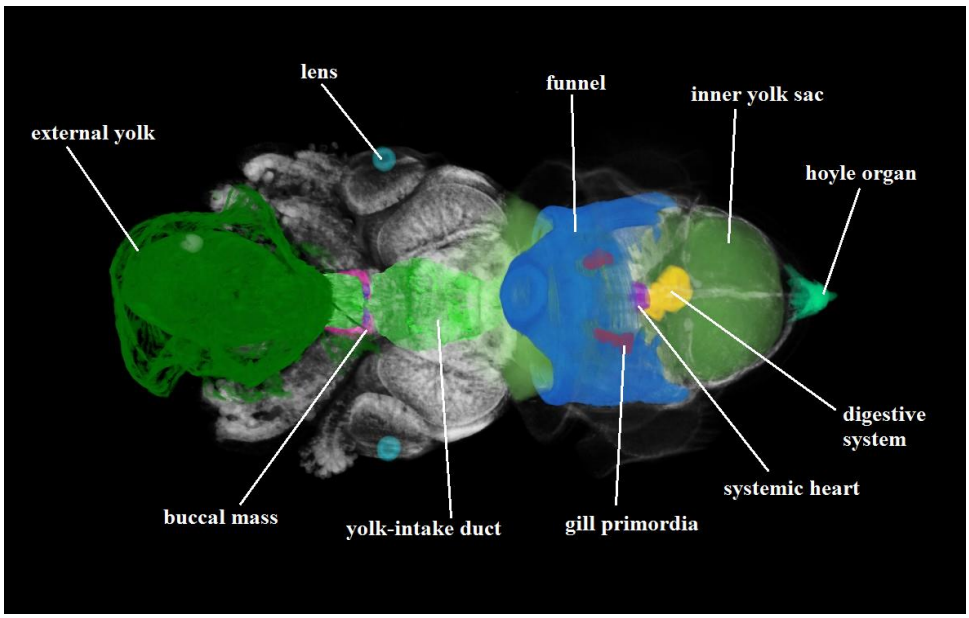
oesophagus is not visible at this stage. Still it is present as indicated by the existence of an oesophagus in the following younger stage 26. The digestive system is visible, though the typical U-shape is not any longer obvious. It is rather comparable to a much flattened V-shape.



Picture 3: Lateral view. The external yolk is about the same size as the head including the tentacles and the yolk-intake duct is organized into two connected sacs where the first sac is smaller than the second and is directly connected to the external yolk. The second sac is larger than the first and is at its posterior end directly connected to the inner yolk sac. The inner yolk sac exhibits three lobes and the border between the former two posterior lobes is still slightly visible.



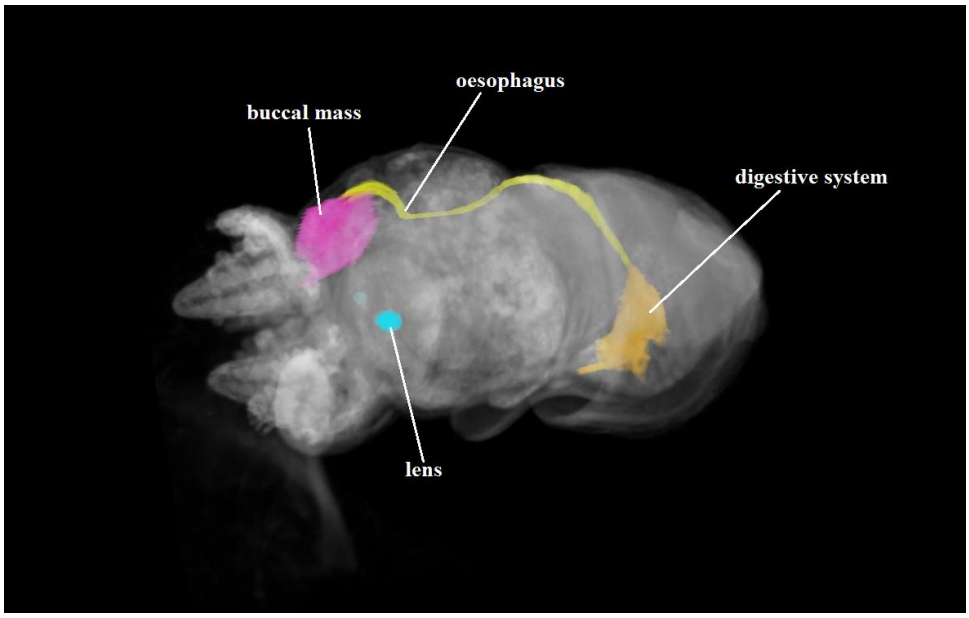
Picture 4: Ventral view. The gills are rather small in this stage and have lost their feather-like shape and structure which could be due to the resolution limits in this scan. Else they have switched their position to a more anterior position which could be due to the smaller size of the body. The body is remarkably slender than the head. The systemic heart is very small now but stays at the same location.Looking at the squid ventrally the width of the yolk-intake duct is noticeable. The organ of Hoyle is also present and forms a spike on the posterior end of the body.



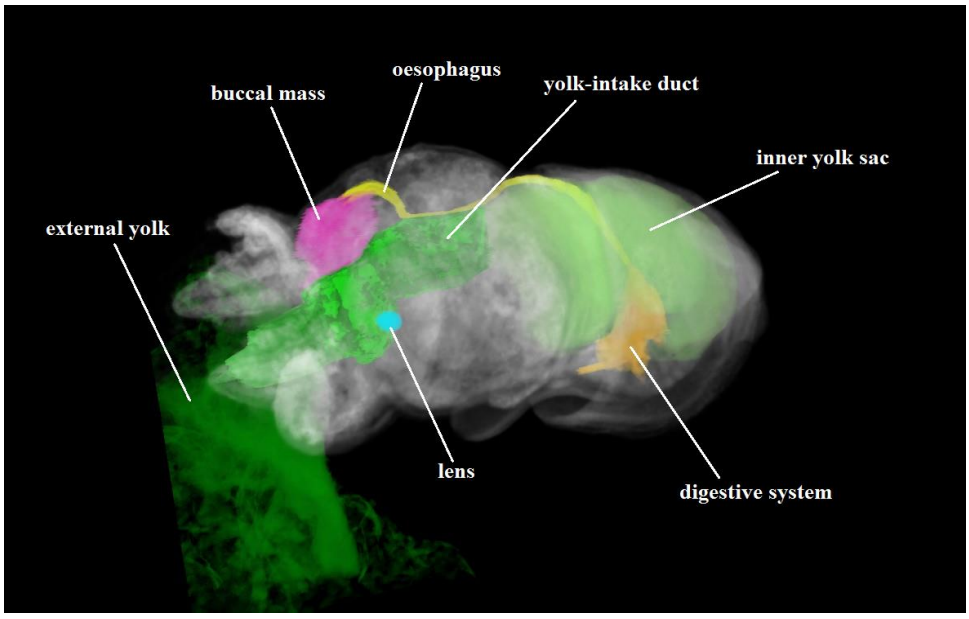
Picture 5: Ventral view. The funnel is about half the length of the body, excluding the head, and its connection to the body cavity is broader than before. Again there is bend of the funnel end.

Stage 26 – Day 17 after fertilization

Total body length: 1.6 mm

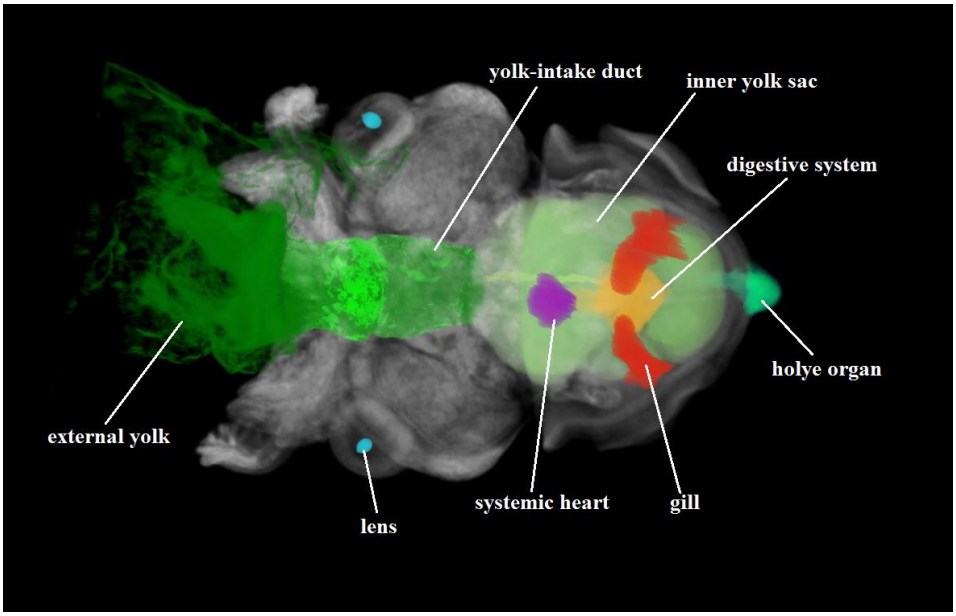


Picture 1: Lateral view. The lenses are rather small. The beak is not observable in stage 26. Only the buccal mass can be determined, it has a flattened, oval shape. The oesophagus is visible and leads from the buccal mass to the digestive system, which is very small and not at all U-shaped.

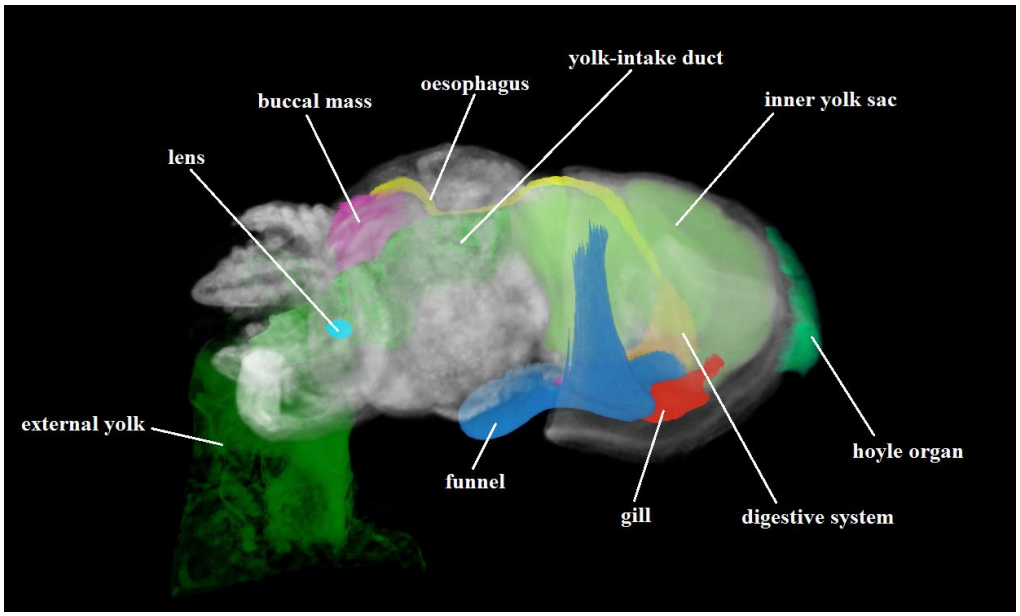


Picture 2: Lateral view. The external yolk is too big to be fully included in a scan with this

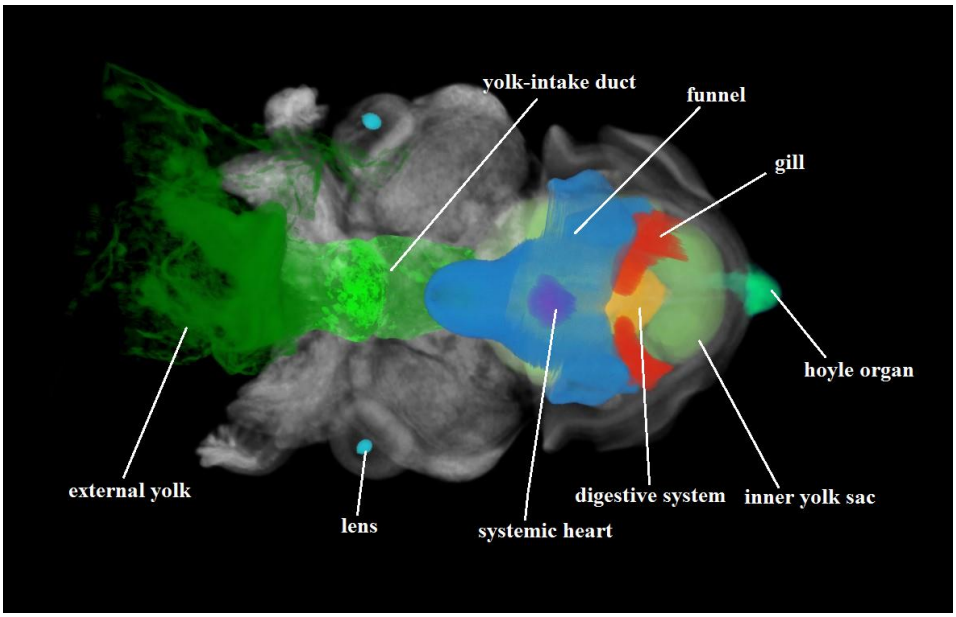
resolution therefore it is only partly visible. The yolk-intake duct is very massive and rather short now and consists of one broad channel connecting the external yolk directly with the inner yolk sac. The inner yolk sac has three lobes and occupies the major part of the body cavity.



Picture 3: Ventral view. The gills are a located in the posterior half of the body and slightly show the feather-like structure that is characteristic for this organ. It is to be assumed that the gills and the heart are at about the same size as in stage 28, since the resolution in this scan is better than in the scan of stage 28 and therefore the structures are better visible.



Picture 4: Lateral view. The Hoyle organ is visible as a flattened spike at the posterior end of the body though it occupies a larger area and seems to be composed of a softer tissue.

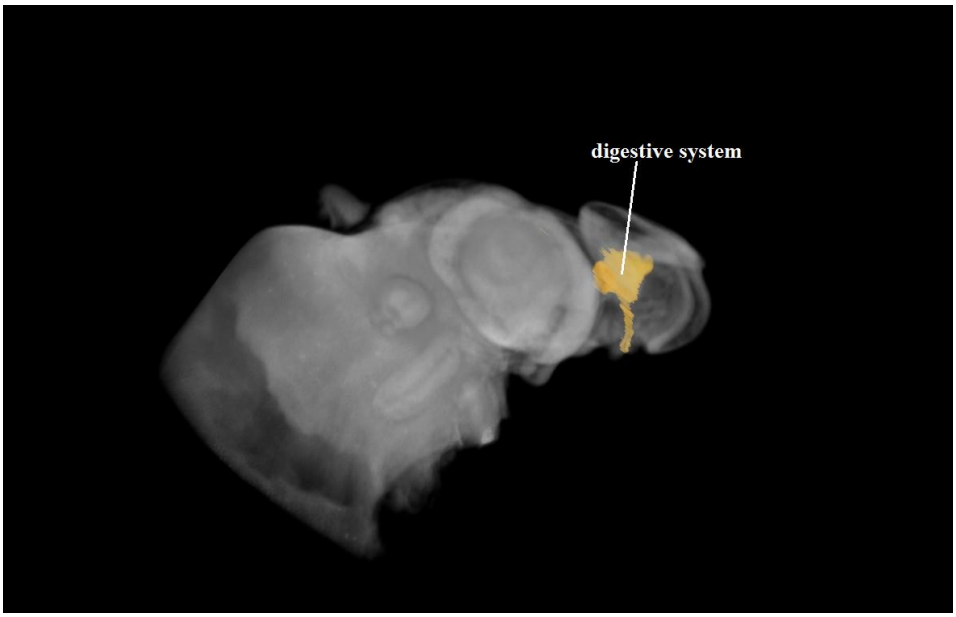


Picture 5: Ventral view. The funnel covers more than the half of the ventral body side excluding the head. Anyway the structure is smaller and covers more of the ventral body side since the body is smaller too. From this ventral point of view it is observable that the body now is remarkably smaller than the head.

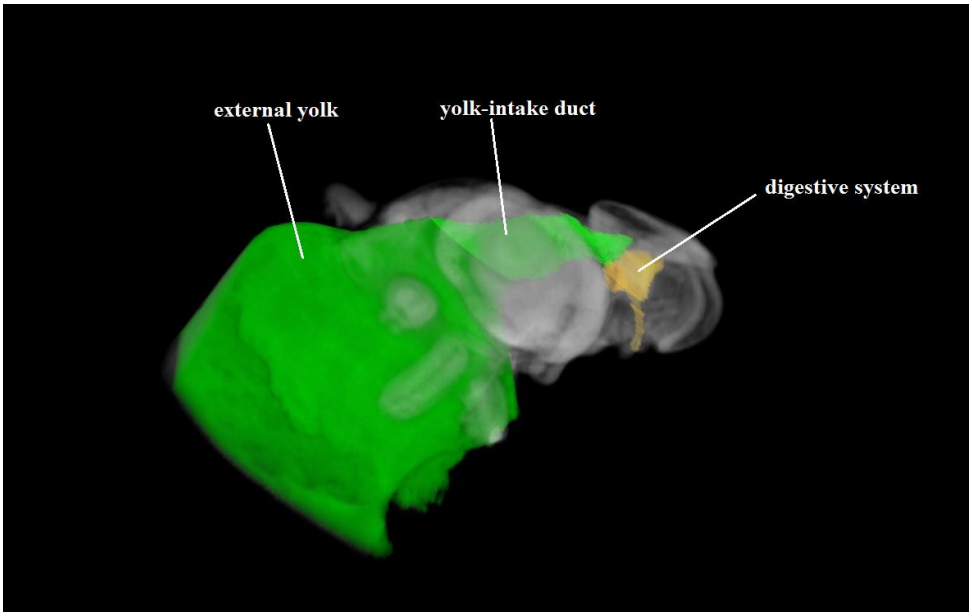
Stage 25/26 – Day 16-17 after fertilization

Total body length: 1.2 mm

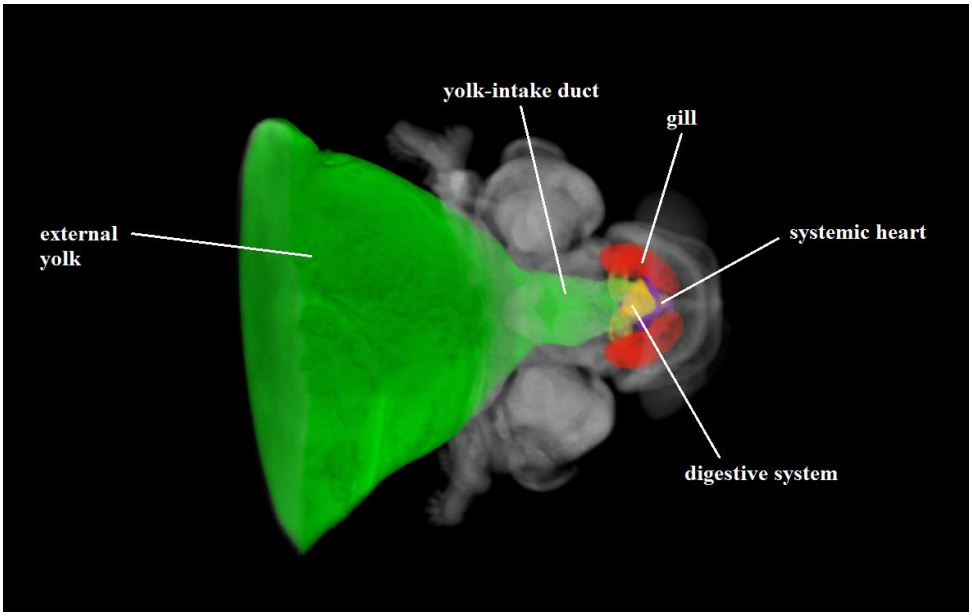
The smaller stages could not be assigned precisely, which is why they are given in-between numbers.



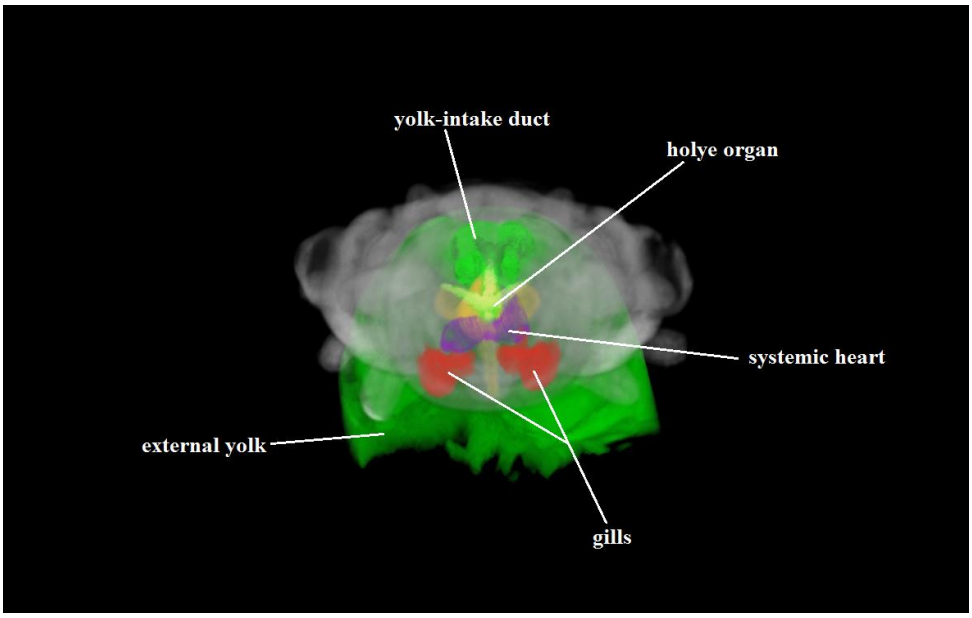
Picture 1: Lateral view. In stage 25/26 there is no eye lens present and also the beak and the buccal mass are not identifiable. The oesophagus is not visible due to resolution limitations, like it was the case at stage 28. In contrast to the oesphagus the digestive system is very well visible. The head is about twice the size of the body.



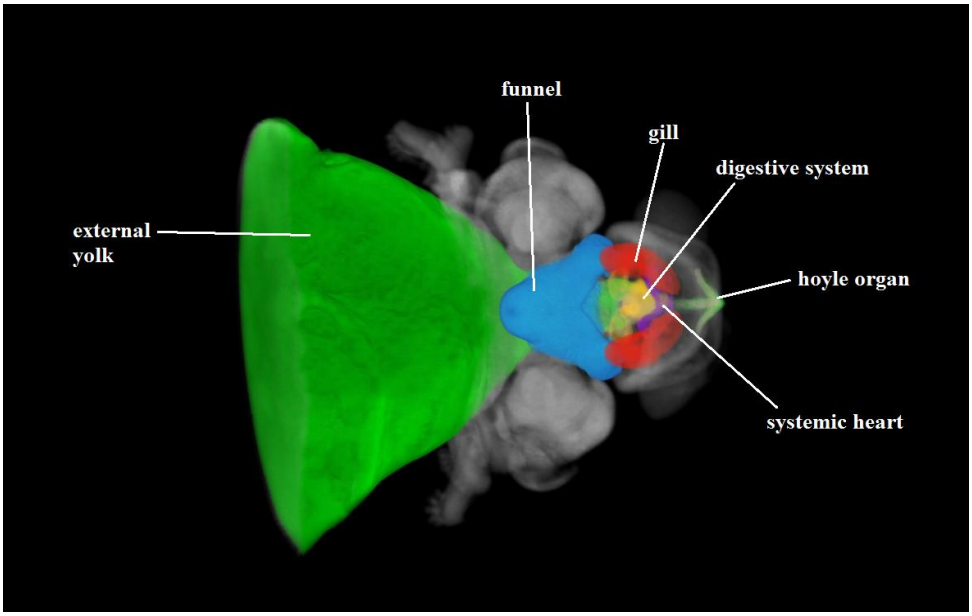
Picture 2: Lateral view. The external yolk is remarkably larger than the embryo and is partly cut away in order to obtain a higher resolution scan of the embryo. The yolk-intake duct equals the one of stage 26. The inner yolk sac is barely there and rather represents the end of the yolk-intake duct. A slight indication of two lobes is visible in picture 3.



Picture 3: Ventral view. The gills are two small patches that are located bilaterally in the ventral half of the body. The systemic heart also is a small patch, located between the gills.



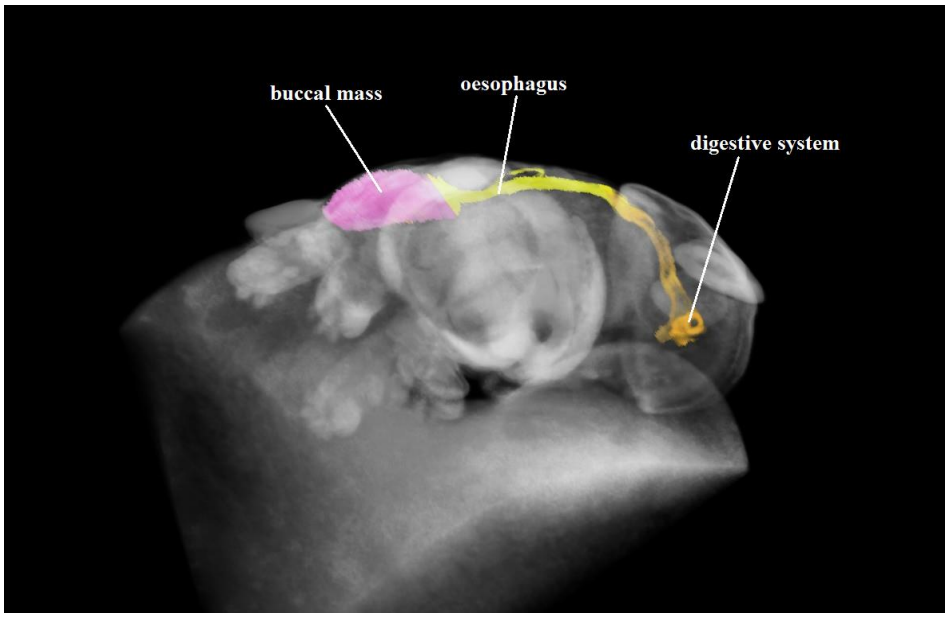
Picture 4: Posterior view. A posterior view shows the anchor-shaped Hoyle organ and also makes the systemic heart better visible. It is lies dorsally of the gills.



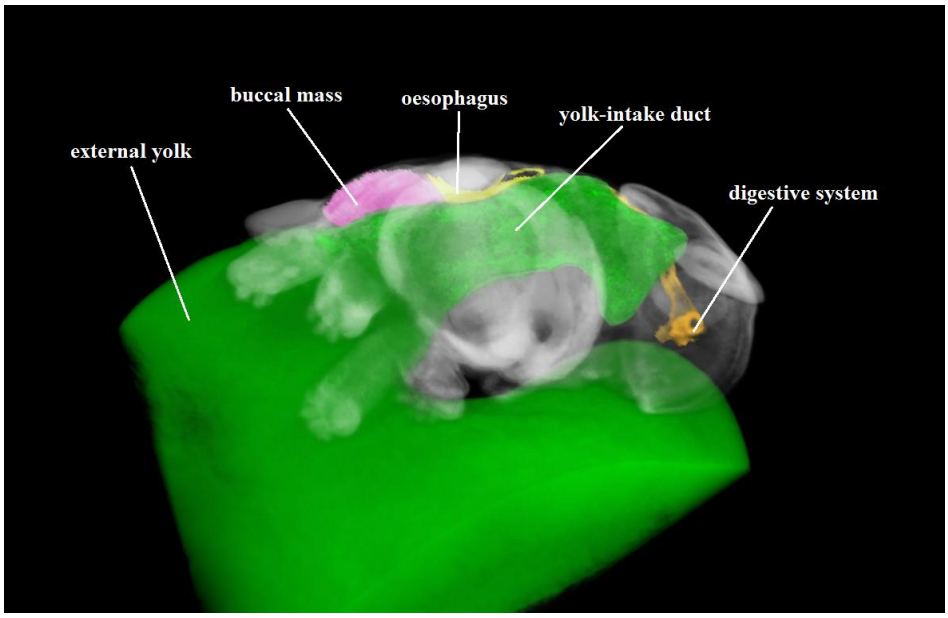
Picture 5: Ventral view. The funnel is very short in this stage, and its two appendages nearly enclose the body cavity dorso-ventrally. Also the funnel is located further anterior than in older embryonic stages and seems to attach between the head and body.

Stage 23/24 – Day 14-15 after fertilization

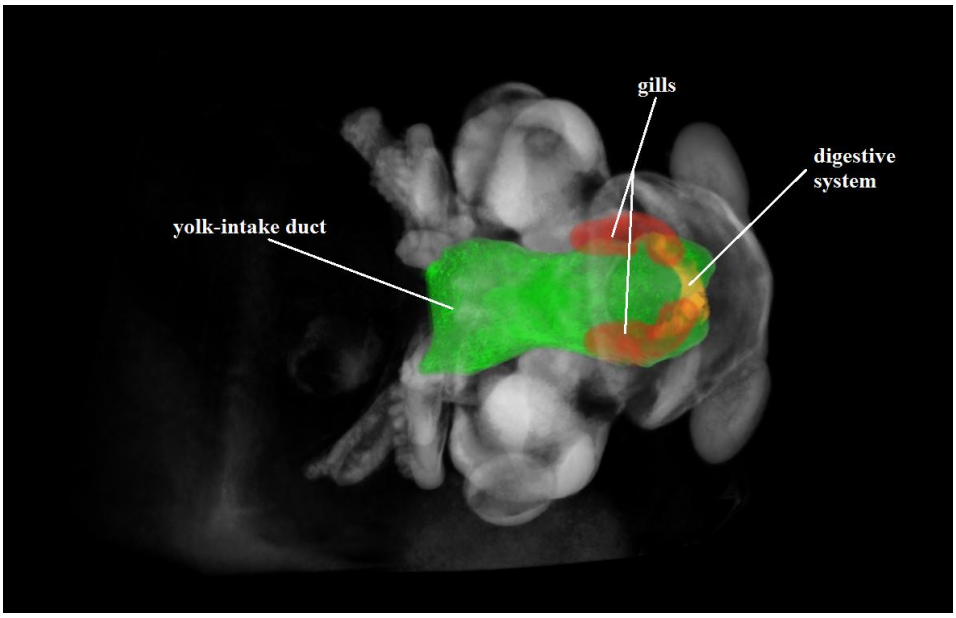
Total body length: 1.2 mm



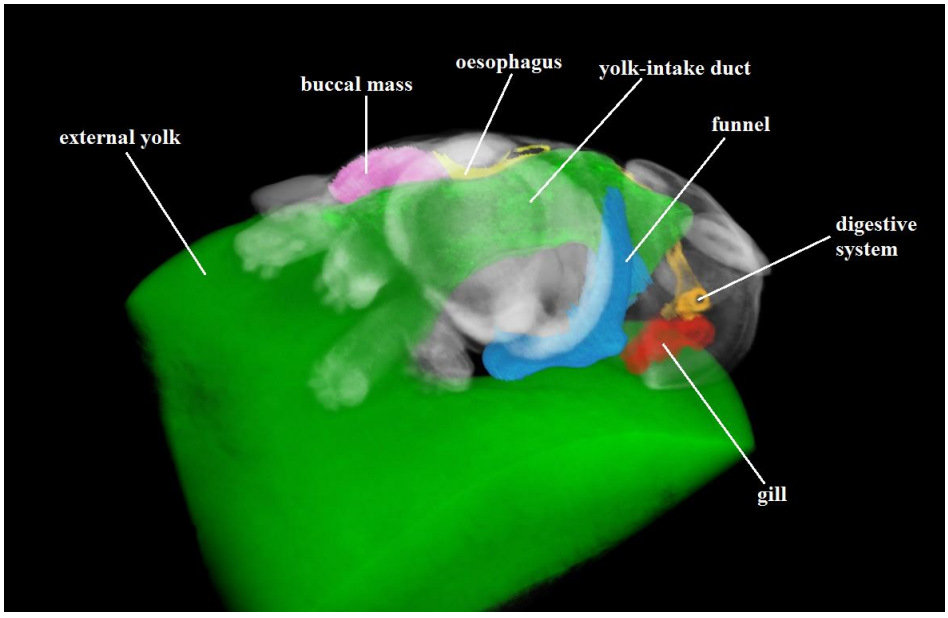
Picture 1: Lateral view. The body is very short and the head is noticeable larger than the body. The buccal mass is visible as a flattened mass on the dorsal side of the head and is followed by the oesophagus. The digestive system exists only in rudimentary form no rectum seems to exist at this point of development.



Picture 2: Lateral view. The external yolk is visibly larger than the embryo. The yolk-intake duct can be equaled with the inner yolk sac at this point.



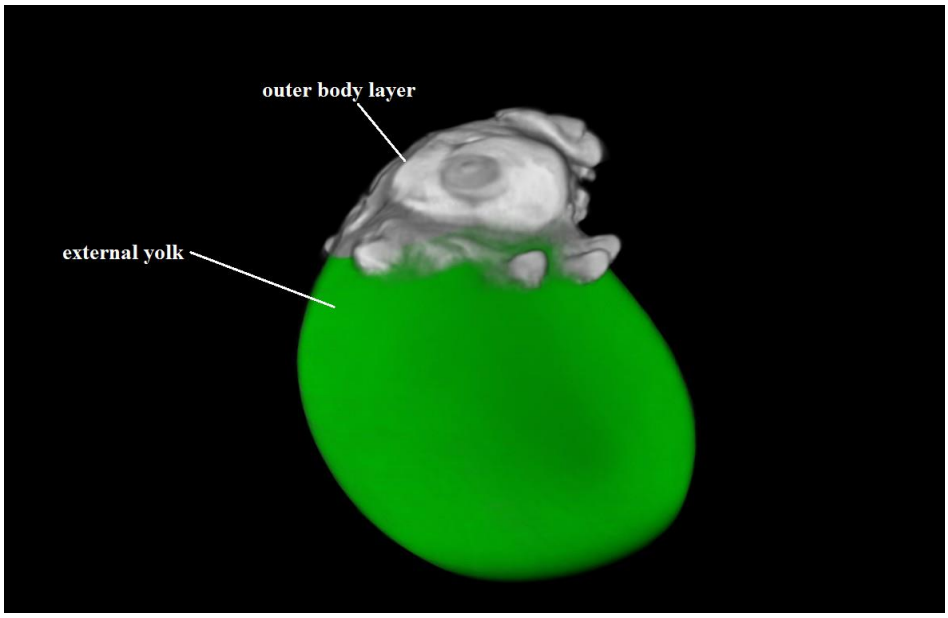
Picture 3: Ventral view. The gills are visible as small, kidney-shaped bilateral structures on the ventral side of the body. A connection seems to be present between the gills and the rudimentary digestive system. The external yolk is masked in this image to give a better view on the embryo.



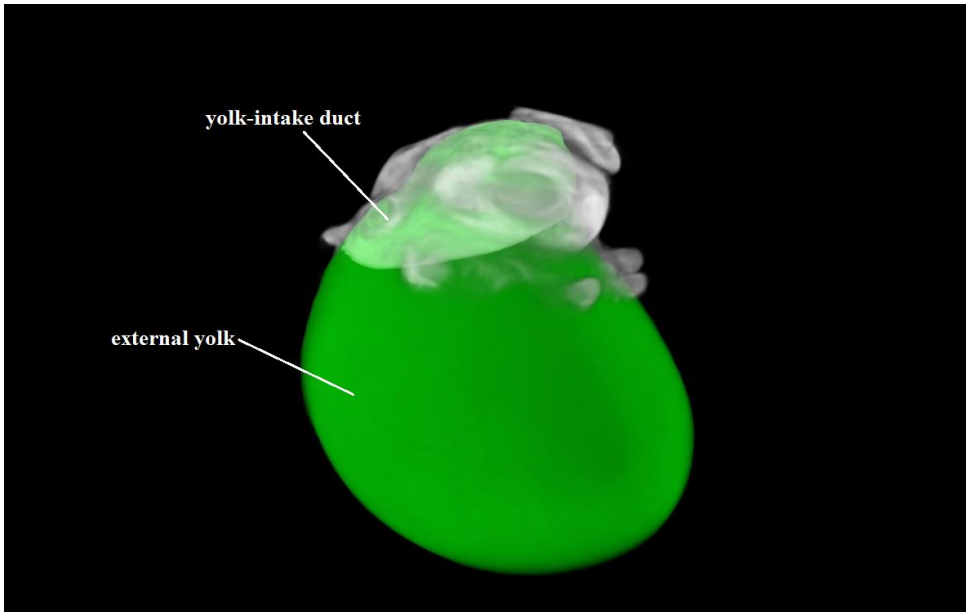
Picture 4: Lateral view. The funnel is consisting of its two appendages, nearly surrounding the body and only a very small knob outside the body.

Stage 19/20 – Day 10-11 after fertilization

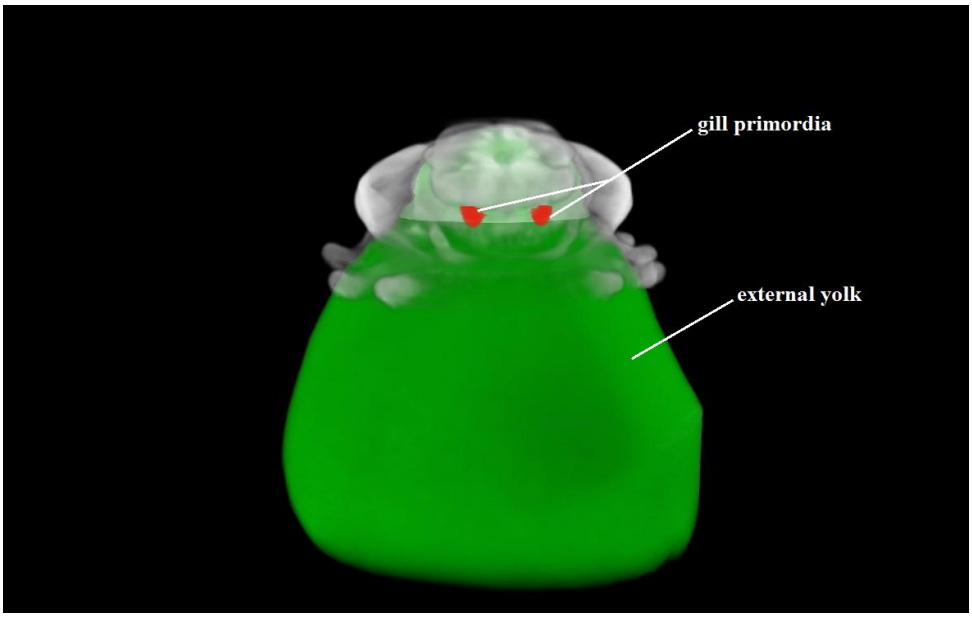
Total body length: 1.1 mm



Picture 1: Lateral view. The outer body layer, whose form already looks like a small squid with very big head, and a very small body as well as the external yolk is visible.



Picture 2: Lateral view. The yolk-intake duct is rather a part of the external yolk, starting to immigrate into the body.



Picture 3: Posterior view. In a posterior view the small gill primordia become visible. They are located under the ventral margin of the developing mantle.

Particular findings

Several embryonic structures could not have been detected without the aid of microCT. For example the radula in the hatchling and in the adult would not have been visible without taking microCT scans. The radula was especially hard to find in the adult, since the full-grown radula seems not to take up the stain and therefore was only visible due to its dense tissue when taking a closer look and a zoomed in scan of the buccal mass. Furthermore the yolk-intake duct would not have been visible at all without taking a microCT scan. It has not been described before in *Euprymna scolopes*. It constitutes a pathway for yolk intake separate from the oesophagus. This has only marginally been described in any other cephalopod until now even though it can be seen in sketches and descriptions from *Loligo sp.* embryos (Fioroni & Meister, 1974).

Non-detectable structures

There is a number of limitations when using microCT. Here too I want to start with the limitations I encountered in the adults and then continue with the embryonic stages, starting at stage 30 going backwards.

I did find the radula in the male adult only after doing a close-up scan because I knew it had to be somewhere since it was visible in the hatching stage without any doubt. I think the stain was not taken up in the adult radula because the chitinous material was too hard for soaking up a stain or it would take an even longer time to stain.
In the female I also could not find the gill heart which is due to the flattening body deformation, which happened when preparing the animal for the first proper scanning.

Some organs could not be found in all the stages. In the case of the ink sac this absence is due to the fact that the animals were stored for an extensive period of time in ethyl alcohol, so there was no natural coloring left, and as the ink-sac without contained ink is just a thin layer of tissue I was not able to find it in the microCT pictures even though I knew where to search.

I could not find the light organ in any of the embryonic stages either. The light organ would have been very interesting to observe in development but it was not visible.

In the earlier stages – younger than stage 25/26 – I could not see the heart, although, according to Lee et al. 2009b, it is already beating in living animals of the same stage.

The online 3D Atlas

Below is an overview table of the 3D online Atlas with thumbnail pictures that provide a direct link to the scanned embryos. [**Fig. 11**]

There are four different types of content. The "Stack" thumbnail pictures lead to the uploaded original microCT stack (TIFF format) of the stage chosen. The "Labels" thumbnails hold a connection to the reconstructed marked and labeled 3D images series (JPEG format) of the chosen stage. The "Metadata" symbols will bear a connection to all information concerning the treatment of the individual. This will be a link to this Master thesis as soon as it is published. The thumbnail "Movie" is only present for the adult male since only for this individual a movie was produced.

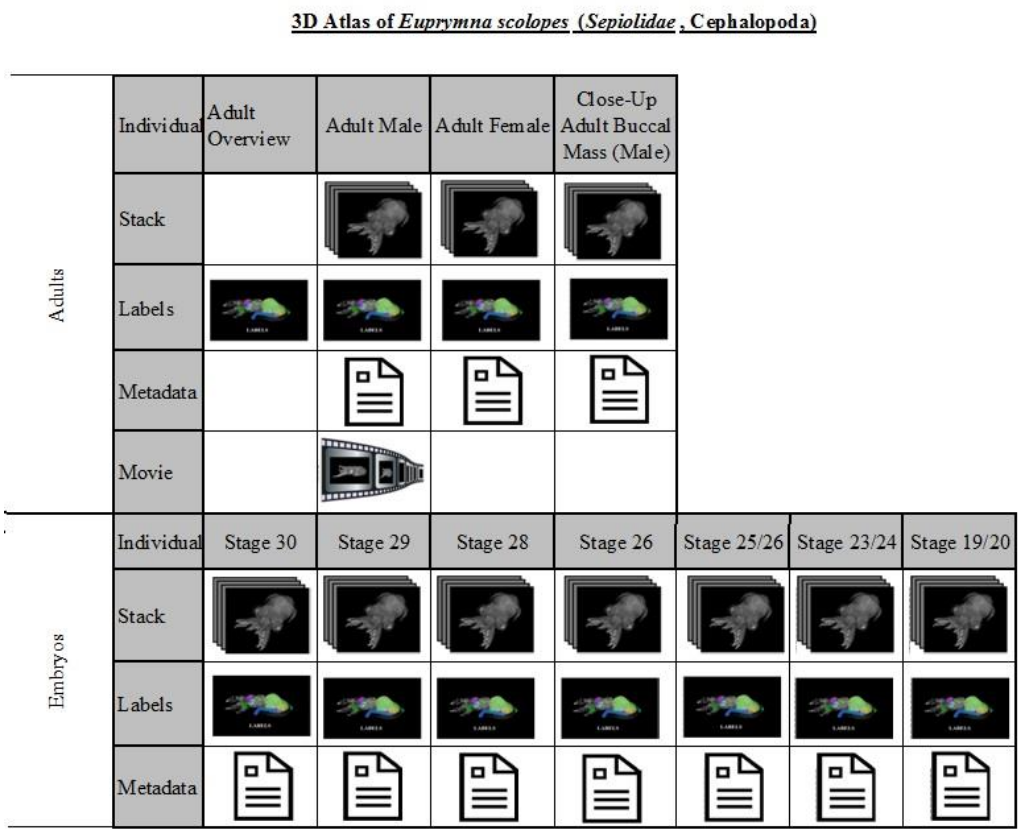


Fig. 11 Screenshot of 3D atlas overview. The thumbnail pictures contain a link to the chosen data set. The stacks lead to the original microCT stacks, the labels lead to the marked and labeled 3D images, and the metadata lead to the informational data for every individual which will be a link to the published Master thesis. A movie was established only for the male adult M2.

Since the deposited links are not visible within this picture the following table [**Table 2**] shows all of them.

Table 2 Listed links to online 3D atlas. Each link leads to the chosen media on the MorphDBase homepage.

Adult Overview marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-24.1
Male adult rotation movie	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-25.1
Male adult original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-35.1
Female adult original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-33.1
Buccal mass close-up original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-34.1
Stage 30 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-26.1
Stage 30 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-36.1
Stage 29 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-27.1
Stage 29 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-37.1
Stage 28 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-28.1
Stage 28 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-39.1
Stage 26 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-29.1
Stage 26 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-40.1
Stage 25/26 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-30.1
Stage 25/26 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-42.1
Stage 23/24 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-31.1
Stage 23/24 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-38.1
Stage 19/20 marked and labeled	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-32.1
Stage 19/20 original stack	http://www.morphdbase.de/?C_Klimpfinger_20150830-M-41.1

The online 3D atlas was released on MorphDBase.de on 30th of August. The uploaded material includes the original stacks as well as a picture series with the marked and labeled organs of each developmental stage and the adults and a rotating video of the male adult. It can be found on the MorphDBase homepage within the tab “Browse Contents” and in the category “Media”. [**Fig. 12**]

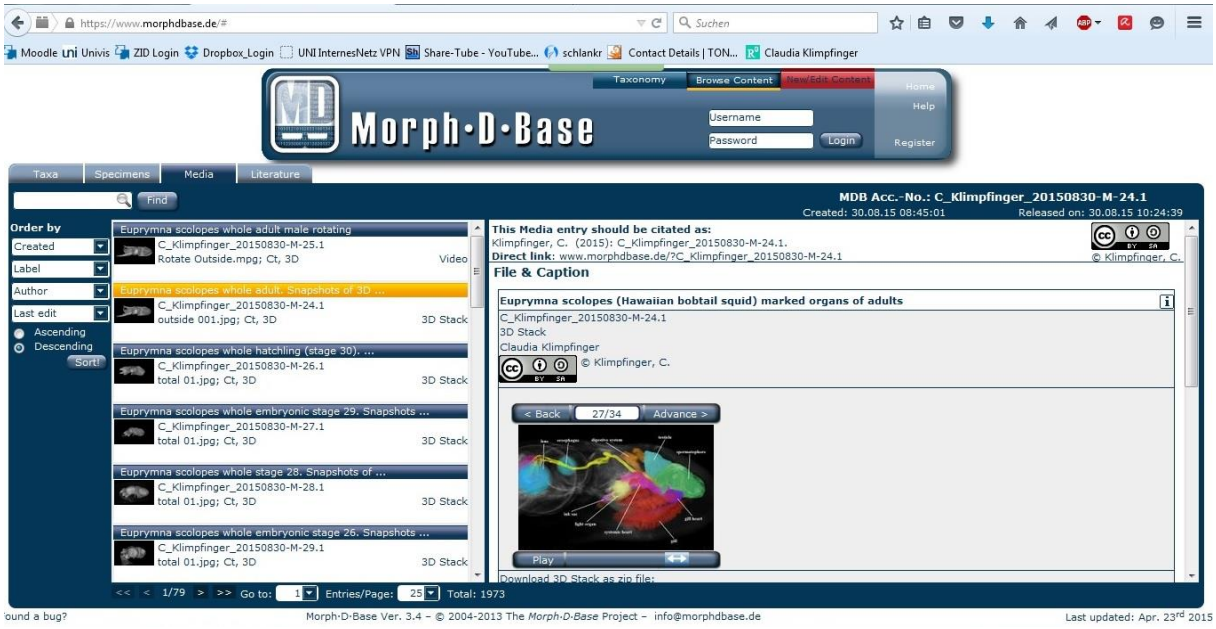


Fig. 12 Screenshot of MorphDBase. The uploaded data include the original stacks as well as a picture series with the marked and labele d organs of each stage. It is found within the tab “Browse Content” in the category “Media”.

The MorphDBase page also provides the possibility to connect content to other pages and articles by simply copying the link stated under “Direct link” and pasting it into the needed position. [**Fig. 13**]

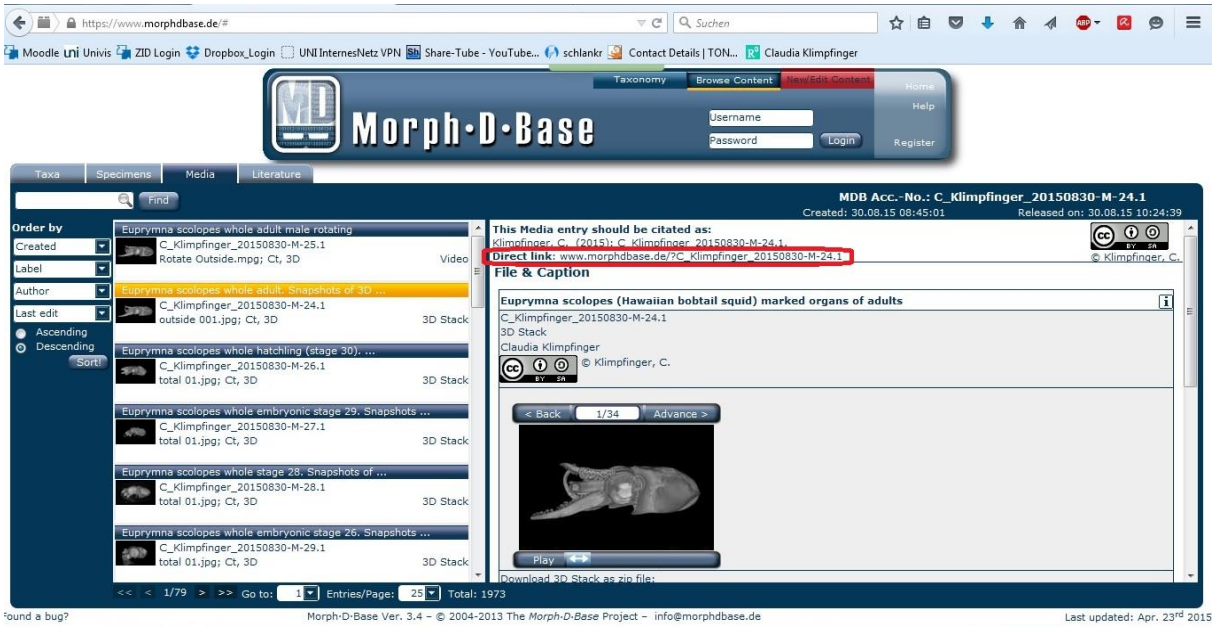


Fig. 13 Screenshot of MorphDBase with marked direct-link option. Copying this direct link to another homepage or an online article makes finding the desired information on MorphDBase easy.

It is also possible to download the microCT stacks – the original as well as the marked and labeled picture series – in different sizes so they would not be the same huge size the originals are. Still, also the original size can be downloaded. [**Fig. 14**]

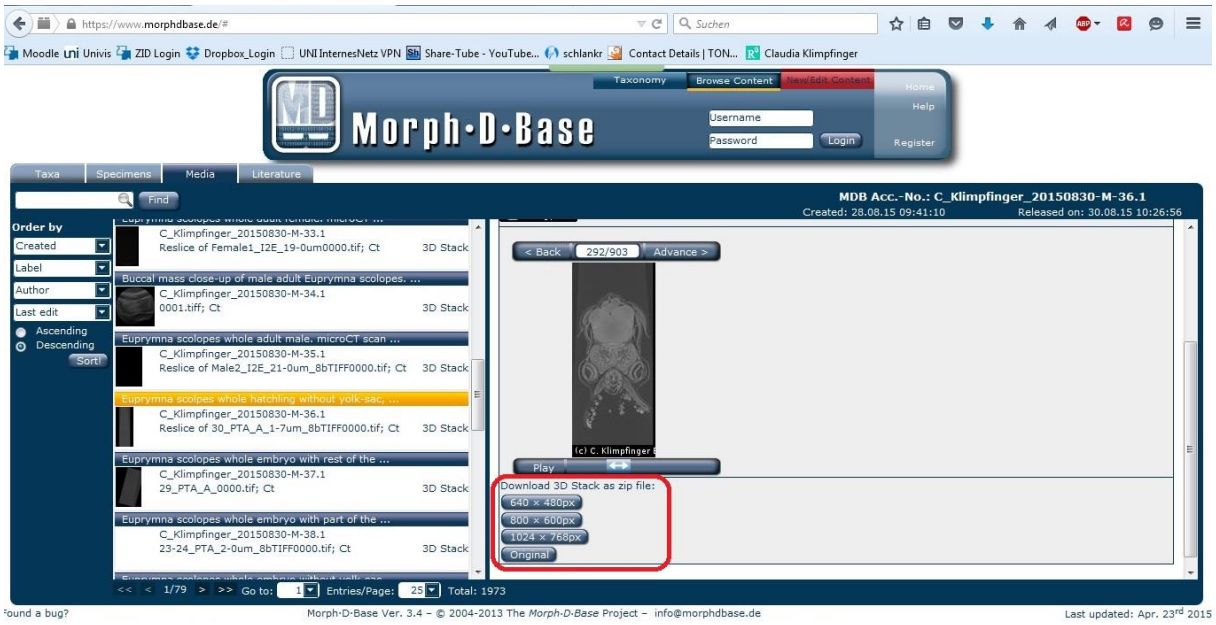


Fig. 14 Screenshot of MorphDBase with download options marked. These download options allow the download of the stack in a smaller size than the original was, but also in the original size. Therefore computers with less capacity can also be used for downloading and saving the stacks.

Another advantage of the MorphDBase web page is the possibility to link associated media to an entry. For *Euprymna scolopes* I always linked the original stacks to the marked and labeled image series with the intention that the image series are found first or are catching more attention due to the bright colors and the descriptions, and then the user, whose curiosity is piqued, can follow the associated media link to the original stacks. [**Fig. 15**]

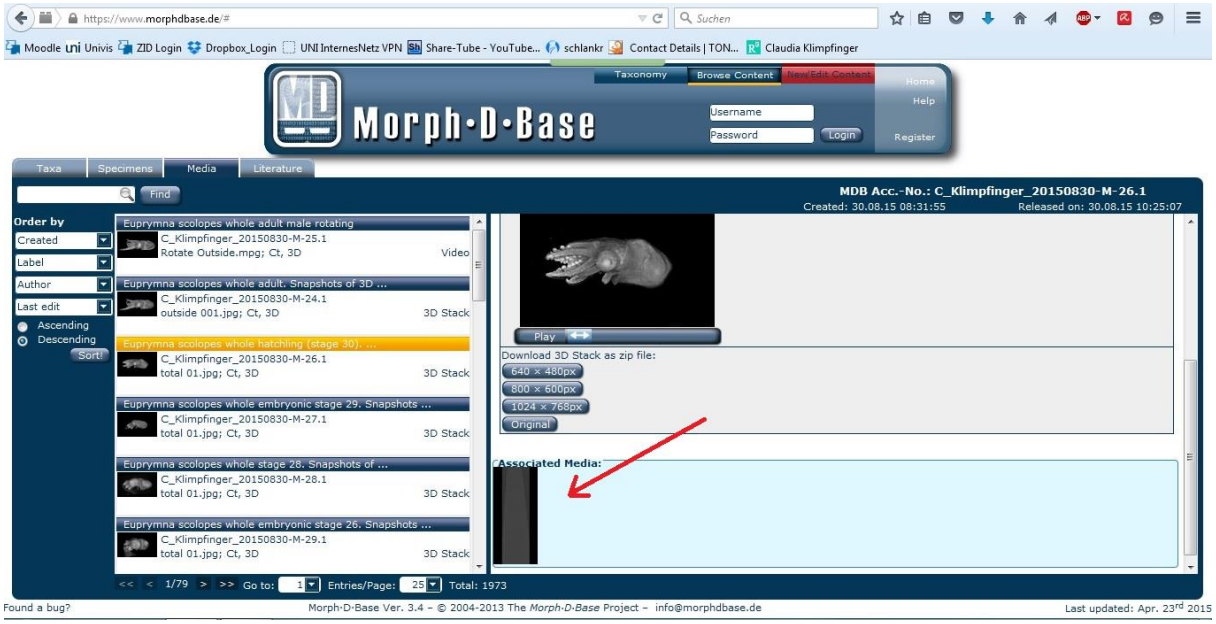


Fig. 15 Screenshot of MorphDBase with marked associated-media option. With this option different materials uploaded on MorphDBase can be connected with each other to make it easier for the user to find all the information concerning one entry.

In addition to the entry in MorphDBase I will enlarge the Wikipedia article of *Euprymna scolopes* in English and German and also include the links to the MorphDBase homepage. The links will be selectable by clicking on a thumbnail symbol which is linked with the corresponding MorphDBase link. The same table of thumbnail links will be included in a future publication in the BMC online Journal. [**Fig. 11**]

Discussion

Contrasting stains for microCT in cephalopod organogenesis research

The comparative test of PTA (phosphotungstic acid) and IKI (iodine potassium iodine) as a constrasting stain for cephalopod embryos and the re-staining of an IKI specimen with PMA (phosphomolybdic acid). In this work it was demonstrated that PTA is better to use on *E. scolopes* embryos, because IKI did not show the organs very well and the contrast was not as good as in the PTA scans. All resulting images were blurrier with IKI. The later test of re-staining IKI with PMA gave the worst results of this comparison. The microCT pictures were very blurry and had very little contrast.

PTA is one of the most broadly used contrast stains for microCT, though it takes a longer incubation time than IKI, because it is a larger molecule. PTA is known to bind heavily to various proteins and connective tissue, which suggests that it is a useful stain for x-ray imaging (Metscher 2009b). The fact that PTA is a larger molecule seems to be the reason why at the hatchling stage the stain did not reach the very center of the inner yolk sac. PTA staining therefore is limited to smaller specimens. In all the other embryos the stain colored the entire embryo and provided a good contrast.

For staining the adults I used IKI in different dilutions first and then changed to I2E (1% iodine in absolute ethanol) to get an even better contrast. Both stains worked out well, though IKI took much longer (21 days) than I2E (8 days) to stain all the tissue. In order to use I2E the storage medium had to be changed step by step to 100% ethanol – each step lasting at least 3 hours – which took another three days. Still, I2E is the faster staining method. The resolution cannot be compared, since it was not the same scanning machine used to scan the animals with the different stains.

Other staining methods were not suitable for *Euprymna scolopes* since either the small size of the embryos or the tissue type that needed to be stained prohibited the use of those other stains. For example osmium staining, which is a conventional fixation and staining method in biological electron microscopy, only penetrates samples up to a millimeter scale thickness. Another way to stain tissues is the impregnation with gold or silver particles, which is a conventional procedure in light microscopy observation of soft tissues. But both, gold and silver staining, tend to visualize only neuronal tissue. This makes them unsuitable for giving

contrast to organs other than the parts of the nervous system (Mizutani and Suzuki 2012).

Resolutions with the microCT

The smaller the reconstructed voxel size, the higher the obtained resolution. The scans were made with different zoom lenses according to the size of the subject. Therefore the results had different voxel sizes. **[Table 3]**

To be able to use a higher resolution for the embryonic stages 29 and 30, we scanned the animals in two halves and stitched the reconstructed halves together again. All the younger stages were small enough to be scanned as a whole with adequate resolution. The adults were scanned with the macro lens and also in two halves and stitched together when reconstructed. The buccal mass close-up was done with a normal lens since it is much smaller than the whole adult squid.

But not only the voxel size is responsible for a good picture, it is also the quality of the scintillator and the lens of the microCT machine that makes a difference. This is very well visible in the resolution size of Male 1 (IKI) and Male 2 (I2E). Except for the different stains they also have been scanned with different machines, and, even though the voxel size of Male 1 is smaller than the one of Male 2, the resolution of Male 2 is much better. [**Table 3**]

The 1x1 binning seems to make the most sense for a good result. As mentioned before, the larger individuals (stage 29, 30) had to be scanned using more than one cycle.

Table 3 Overview of the voxel sizes of scanned subjects. All of the given sizes are reconstructed voxel size stated in µm.

Stage	Reconstructed Voxel size [µm]
F1 (IKI)	20.9
M1 (IKI)	20.9
F1 (I2E)	19.0
M2 (I2E)	21.0
Buccal mass close-up M2 (I2E)	4.0
30	1.7
29	1.7
28	1.7
26	2.0
25/26	1.6
23/24	2.0
19/20	1.7

MicroCT – a new approach to embryo research

The work with microCT requires the fixation of the animals and also their staining, which is necessary for giving good contrast of the tissues within the body, especially within non-skeletal organisms such as mollusks. There are some advantages to microCT. The animals are observed at very high resolution and a higher magnification is possible than with the dissecting microscope. Also the possibility of studying the animal slice per slice and virtual reconstructing the different plains afterwards provides new opportunities for finding undescribed structures – such as the yolk-intake duct and the radula in this case. The reason why I think microCT is a very good tool for the study of organogenesis in development is the unique, illustrative and comprehensive results obtained from such a project. Colored and labeled pictures, that can be taken apart and put back together, are by far better suited to show results than just descriptions and pictures from the outside – even when the outside is quite transparent. In addition microCT provides the possibility of taking measurements and thus facilitates a quantitative embryology.

Newly observed structures of cephalopod development

Several barely described structures could be found in this species: such as the yolk-intake duct. At my first attempt of interpretation I thought it has to be the oesophagus that connects the external yolk with the inner yolk sac, and that the inner yolk sac already is located in the future digestive system. But by the time I labeled the beak and the buccal mass and found the real oesophagus located dorsally of the previously assumed “oesophagus” it was clear to me that this structure cannot be the oesophagus but has to be something different. I scoured the already existing papers dealing with *Euprymna scolopes* for a hint, without finding any reference to it. When not being successful with *E. scolopes* papers, I widened my search to other cephalopod species. In a paper from 1974 on the embryology of *Loligo vulgaris Lam.* (Fioroni and Meister, 1974) I found a short description and sketches of a “yolk shaft” (German: Dotterstiel) present during development. This yolk shaft connects the external yolk and the inner yolk sac and narrows during proceed of development. It is located parallel to the oesophagus. The entire yolk is surrounded by a special yolk epithelium or yolk syncythium, passing on nutrients from the yolk to the blood vessel system, and therefore also is important for the respiration during embryogenesis.

It is also stated, that the foregut (German: Darmrohr) develops separate from the yolk epithelium. Due to this information I recognized that the structure I found in *E. scolopes* was comparable to the described structure of *Loligo vulgaris*. Therefore I called it yolk-intake duct.

Fioroni and Meister (1974) describe the reduction of the inner yolk sac after hatching. Due to their findings the inner yolk sac diminishes simultaneously with the growing of the midgut gland. It is to be studied if this is the case in *E. scolopes* too, since no midgut gland could be identified. Probably the structure labeled with "Caecum?" in the sketches of the dissection could be the midgut gland, but this is to be tested (maybe by histological sectioning). Nowhere else than in the publication of 1974 by Fioroni and Meister such a yolk-intake duct or yolk shaft is described for cephalopods. This is maybe due to the fact that organogenesis is not described for many cephalopod species, and even the topography of organs in the adult is not described for many species. The two separated pathways (oesophagus and yolk-intake duct) are very well visible on figure 10. [**Fig. 10**]

Also the radula probably would not have been found without using microCT. I first saw it in the scan of stage 30. The radula has not been described in any publication concerning the development of *E. scolopes* before. Dissecting the adult I already knew there had to be a radula. It was very small and hard to find even though I precisely where to look. In the microCT scan of the adult I also had to search for the radula, since I knew it was there. Without paying particular attention to this structure, the radula is barely visible in the adult. In the embryonic stage 30 the radula was only visible with of the aid of the microCT. As the radula in the adults did not take up any stain, I do not know how likely it is to find the radula in traditional histological sections, even when combined with immunostaining.

Difficulties with microCT diagnosis

Compared to the living individuals observed under a dissecting microscope, the microCT has some disadvantages too. The most obvious one is the difficulty of tissue distinction. The pictures made by the CT scanner are all grayscale; there is no movement and no natural pigmentation visible. It is hard to identify organs and sometimes it is also hard to define the borders between organs. Everything that is beyond the maximum resolution is hard to identify due to blurriness in the magnified pictures. In any case it is a good idea to obtain an overview first. We used Amira for the reconstruction of the specimen, but Amira only shows diagonal

cuts of the scanned animal. This makes it necessary to use another program for an overview. I used ImageJ which shows straight cuts of the animal.

The next difficulty is the missing pigmentation – both natural and artificial. By artificial staining I mean immunostaining to show different types of tissue in different colors. Under the dissecting microscope natural and artificial pigmentation are visible. Also in microCT no movement of the animal itself is possible. Movement would even be disadvantageous in microCT, but under the dissecting microscope movement can, in some cases, be helpful when searching for a specific structure, for example the heart, which would be beating in a live animal.

Competing methods for 3D image generation

There are many different ways to generate 3D images from biological objects. The oldest method of presenting results in a three-dimensional way is the reconstruction of serial sections. During the late nineteenth century, in the beginnings of 3D reconstruction, those reconstructions were produced manually from physical serial sections by the stacking of cut-out wax plates (Born, 1883).

In modern times 3D reconstructions are generated by computers that generate virtual three-dimensional images. To present histological data in 3D a surface rendering, based on image segmentation, is the most common way to do this. Another possibility is the volume rendering of serial sections, which fills a gap in the field of 3D micro-anatomical visualization because it combines 3D imaging and histological sections (and the information such sections contain, for example, tissue identification) (Handschuh et al. 2010). Confocal laser scanning microscopy (CLSM) produces 3D images by generating optical sections through an object. Even whole-mount fluorescence preparations with different stainings in one object are possible and enable the generation of high-resolution images. Recordings on such whole-mount objects are limited to object-thicknesses of about 100µm (Wanninger 2007). Sands et al. (2005) described good resolutions from volumes in the range of 1µm up to 4mm^3 voxel size.

Today, the most common ways of generating 3D pictures is by different tomographic methods. They are used for soft body-parts as well as for molecular signals and hard body-parts in biological samples. The most common one is the magnetic resonance imaging (MRI)

or the microscopic magnetic resonance imaging (microMRI). One of the biggest benefits of MRI is the capability to take scans of untreated specimens (Handschuh et al. 2010). With special genetic constructs also changing gene expression patterns can be shown in living individuals (Louie 2000). The MRI and microMRI in theory should be able to generate resolutions down to 10µm, but in practice these resolutions cannot be reached. A way to circumvent this problem is to use a more recent form of tomography: the optical projection tomography (OPT). OPT is capable of higher resolutions and also enables the generation of optical colored or fluorescent stained images showing selected tissues (Sharpe 2004).

Still microMRI/MRI and microCT currently can be considered best high-resolution, non-invasive technologies for imaging whole specimens at the centimeter scale and beneath. Freshly fixed specimens as well as museum material, which should not take any harm, can be scanned with both of these methods since they are non-destructive. MRI is better to show soft tissue whereas microCT shows hard structures better. Both methods can be expanded to both, hard and soft tissue, by using chemical stains (Ziegler 2012). Also, those methods bring faster results since conventional histological or ultrastructural methods usually take days or weeks to show first 3D results. Also the preparations for those conventional methods may alter or destroy the studied objects. Another advantage of both of these methods – microCT and MRI/microMRI – is the broad availability of the required instruments as well as the possibility to scan a large number of specimens in a relatively short time. In terms of resolution both methods are very good but still lie below light microscopy and electron microscopy (Ziegler et al. 2010).

Combining several methods of the above named is called "multimodality". This approach to generating three-dimensional pictures with high resolution on specific parts has already been used in some studies (e.g. Forbes et al. 2006; Handschuh et al. 2013).

3D online atlases and possible improvements

The – still rather small amount of –publicly available digital morphological data unfortunately is scattered throughout the internet on several different web pages and databases (like DigiMorph, MorphDBase, MouseAtlas, etc.). All of those pages are maintained by different institutions or working groups and often also have different aims and different financial constructs. Sometimes it is impossible to upload all the data of one project to one page (due to the different types of data) and therefore the data has to be uploaded on different web pages

which makes a direct comparison of these and other data concerning the same species more complicated (Ziegler et al. 2010). Interactive visualization, such as real time manipulation of image datasets, often is possible only when downloading the data, or it is simply not available at all.

Compared to other existing online 3D atlases of embryological model organisms, such as the mouse or the zebrafish, the 3D online atlas produced in this Master thesis is rather rudimentary. Still it has some advantages compared to other atlases. Most 3D atlases found online cover different image producing methods on the common house mouse *Mus musculus*.

Some of the atlases can only be accessed with an account of the university which produced the atlas, as it is the case with the zebra fish browser of the Univeristeit Leiden (http://bio-imaging.liacs.nl/atlasbrowserstart.html). Others seem to be expired as the links stated in the papers do not lead to an online 3D atlas (DeLaurier et al. 2008) but to the general homepage of an institution. In the paper of Belmamoune and Veerbek (2007) there is not stated a link at all even though a 3D online atlas is described. Also in the earliest paper producing an online atlas no link is embedded, but this probably is due to the fact that it was produced in 1996 (Williams and Doyle 1996).

In the Bio-Atlas page of the Pennsylvania State University, a lifespan atlas of the zebrafish *Danio rerio* with 2D and 3D anatomical reference slides is announced, but it is not possible to find any 3D reconstructed pictures. There is a table showing the different developmental stages and the pictures possible to view. Only some of the stages have annotated images. There is a virtual slide technology embedded in the page which is very appealing and it is also possible to view a side-by-side comparison of the microCT virtual sections and the histological sections (http://bio-atlas.psu.edu/zf/index.php).

On the FishNet website of MONASH University an online anatomical reference for zebrafish larval development is provided based on optical projection tomography (OPT) slices of very high quality. Scanned embryo sizes reach from 5 mm up to the adult zebrafish. The 3D models are only visible after downloading and uncompressing the gzip data. Volume renderings of the scans open up in a separate window when clicked at. Those volume renderings are probably supposed to be rotating movies, but as far as I could check on them they were only visible as single pictures captured from different angles (http://www.fishnet.org.au/). In a paper of 2007 Bryson-Richardson, Berger et al. have

documented the establishment of FishNet, including also the rationale of establishing such a web site.

The Edinburgh Mouse Atlas Project (EMAP) is a very well structured page. A stage selection is available in every menu. It is a separate established web page for the atlas data of the mouse. It contains high resolution pictures of wax sections with a thickness of 7 µm and stained with Haematoxylin and Eosin. Out of those wax sections the 3D reconstructions were generated producing surface rendered 3D models. There is a rotating movie for each stage and a Theiler stage definition table containing the stage-number, a sketch of the embryo, the anatomy ontology, and descriptive text. Else there is a link to the Kaufmann interactive atlas (an online atlas with different histological sections) and an interactive anatomy browser (with the possibility to search for genes expressed in the chosen region) embedded in the web page (http://www.emouseatlas.org/emap/ema/home.php). The EMAP is mentioned amongst other web pages in a summarizing paper by Hawrylycz et al. (2011).

Dogdas et al. (2007) established an enlarged 3D online atlas of the mouse showing cryosection data as well as Positron Emission Tomography (PET) and microCT datasets (http://neuroimage.usc.edu/neuro/Digimouse). All of the data is visible only by downloading the datasets and opening it with the BrainSuite program (which has to be downloaded and installed first). The 3D data provide a voxel size of 100 µm. In contrast to the other online atlases existing until 2007, this one is not restricted to a specific organ or the embryonic stages only.

Another way of generating 3D data was used in the Caltech µMRI Atlas of Mouse Development (http://mouseatlas.caltech.edu/index.html). As the title suggests this developmental atlas was produced by using µMRI (micro magnetic resonance imaging). To view the atlases it is necessary to download and install the program MBAT and then download the data files. The labels and colorings are visible when downloading the label-file too, but at least in the case of this online atlas a gallery provides an overview of the images included in the database. There are also links to other mouse atlases and to a YouTube video embedded in the page. The original image stacks are supposed to be opened with open access programs such as Fiji but, in my experience this it did not work because the files in the downloaded dataset where no TIFF files. The establishment of this atlas was documented in the paper of Dhenain, Ruffins and Jacobs (2001).

The advantage of the MorphDBase homepage compared to those other online atlases is the possibility to see the 3D reconstructed marked and labeled images as well as the original microCT stacks without having to download them. Of course there is the possibility to download the datasets in different sizes as a zip file. Disadvantageous are the missing connections to other materials, such as histological sections and gene expression signals. Since there are no histological sections uploaded as an atlas by now there cannot be a link for this kind of data. Since links can only be made to other contents of MorphDBase also the genetics cannot be added. To create a better online atlas it would be necessary to create a separate homepage for all the data. The voxel-sizes provided within this atlas are smaller than the ones in all the atlases mentioned before.

The closest to perfect online 3D atlas would combine the depicting functions of MorphDBase with the advantages of a separate homepage for each species or a distinct group of species where links to different datasets and references can be included and descriptions and navigation can be designed in a simple way in order to make this page the optimal teaching tool for anatomical and morphological issues. Additionally an overview navigation page of all morphological datasets (including links to those datasets) would make a global linkage of digital morphological data much easier.

Summary

MicroCT imaging provides detailed representations of embryonic structures and of structures in whole animals without harming the object, as it is a non-invasive method. Compared to traditional methods microCT scanning has many advantages, such as:

- high resolution in mm and µm dimensions
- the generation of 3D images
- the illustration of structures not visible from the outside

At the same time the microCT method also has some disadvantages, such as:

- the lacking movement of organs in scanned animals
- the necessity for the scanned objects to be fixed

The embryonic structures made visible by microCT scanning are easy to identify due to the possibility of volume rendering, showing the nearly transparent outer body as well as the colored organs simultaneously. Since those results are also pretty to look at, they might catch the attention of people not interested in anatomy initially and provide ideal materials for teaching.

The major results of this thesis are:

- the development and optimization of contrasting procedures for the microCT scanning of cephalopod embryos
- the establishment of an online 3D atlas of *Euprymna scolopes* containing two adults (male and female) and the developmental stages 30, 29, 28, 26, 25-26, 23-24, 19-20

The major biological findings are:

- The description of separated pathways of the oesophagus and the yolk-intake duct in *Euprymna scolopes*
- The observation of the radula, both within the adults and the hatchling, which probably would not have been possible using traditional image generating methods

- The topography of organs in the adult male and female animal.

Still there are open questions left. For example: What is this big structure on the dorsal body side of the adults (in the embryos it is located between the lobes of the inner yolk sac)? Is it really a digestive gland or is it something different?

The open access atlas of *E. scolopes* development allows everyone interested in cephalopods in general, and in *Euprymna scolopes* in particular, to use the data and the 3D reconstructions of the organs in the embryonic stages as well as the ones of the adults. The atlas may also be used in the teaching of cephalopod morphology.

Acknowledgements

In the first place I want to thank Dr. Brian Metscher for encouraging me to do this project and always helping me with new ideas and all the technical stuff. Also I would like to thank the Department for Theoretical Biology of the University of Vienna and its head Prof. Müller for giving me the opportunity to use the microCT of the Department and for including me to the Department so kindly.

A special thank you goes to Athena Copenhaver and Chris Payne from the Montenary Bay Aquarium in California, who helped me getting some adults of *Euprymna scolopes*.

Furthermore I want to thank my family and friends – especially my best friends Katrin Praschl-Bichler and Julia Ulrich and my mom Dipl. Ing. Elisabeth Klimpfinger – for encouraging me and keeping me going.

Glossary

Amira	reconstruction and labeling software
Annotated	Comments provided
Buccal Mass	muscles surrounding the beak and mouth
Cephalopod	All members of the class mollusca, including squids, octopuses, nautilus and cuttlefish
Chitinuos/ Chitin	Long chain polymer structure that usually forms the exoskeletons of arthropods
Cryosection	Frozen dissecting procedure
Dispatched	Killed
Endemic	Existing only on one place in earth
Eosin	A fluorescent compound that binds to muscle fibers
Ganglionic placodes	Thickening epithelium of the developing nervous system
Ganglion	Cluster of nerve cells
gzip	Another form of zip-file, a compressed data file
Haematoxylin	A compound that binds to DNA and RNA and marks cell nuclei
Histology	The study of microscopic anatomy of cells and tissues
Hoyle Organ	Hardened tissue at the hinder (posterior) end of the hatchling, which is needed to puncture the egg shell when hatching
Immunostaining	Antibody-based method to detect specific proteins in a sample
Interpolation	Constructing new data points within the range of known data points
Lateral	Referring to one side (left or right) of the body
microCT (µCT)	x-ray scan for very small sizes
Oesophagus	that part of the digestive system which connects mouth and stomach (German: Speiseröhre)
Organogenesis	development of organs (mostly happening during embryonic stages)
Phylogenetic Tree	Branching diagram showing evolutionary relationships between species
Radula	Chitinous structure in the mouth which helps to transport and mince the food. Also known as rasper tongue (German: Raspelzunge)
Scintillator	Layer in the microCT that converts x-ray to light to make a picture visible to the camera inside.
Surface Rendering	Generating an image of a model showing only the outside surfaces looking like a plasticine model
Ultrastructure	Nanostructure of a biological specimen
Volume Rendering	Generating a 2D projection out of a 3D dataset
Whole mount	Using the whole specimen at once

References

Arnold, J. M., C. T. Singley & L. D. Williams-Arnold (1972). **Embryonic Development and Post-Hatching Survival of the Sepiolid Squid *Euprymna scolopes* under Laboratory conditions.** *The Veliger* 14(4): pp. 361-364.

Belmamoune M. and Verbeek F. J. (2007). **Developmental Anatomy Ontology of Zebrafish: an Integrative semantic framework.** *Journal of Integrative Bioinformatics* (2007), 4(3):65

Born G. (1883). **Die Plattenmodelliermethode**. *Archiv für mikroskopische Anatomie* (1883), 22:584-599.

Bryson-Richardson R. J., Berger S. et al. (2007). **FishNet: an online database of zebrafish anatomy.** *BMC Biology* (2007), 5:34, doi:10.1186/1741-7007-5-34

Caltech µMRI Atlas of Mouse Development: http://mouseatlas.caltech.edu/index.html - last check 6.9.2015

DeLaurier A. et al. (2008). **The Mouse Limb Anatomy Atlas: An interactive 3D tool for studying embryonic limb patterning.** *BMC Developmental Biology* (2008), 8:83, doi:10.1186/1471-213X-8-83

Department of Theoretical Biology, University of Vienna: http://theoretical.univie.ac.at/ - last check 14.9.2015

Dhenain M., Ruffins S. W. and Jacobs R. E. (2001). **Three-Dimensional Digital Mouse Atlas Using High-Resolution MRI.** *Developmental Biology* (2001) 232, pp. 458-470, doi:10.1006/dbio.2001.0189

DigiMorph: http://digimorph.org/index.phtml - last check 8.9.2015

Digimouse: http://neuroimage.usc.edu/neuro/Digimouse - last check 8.9.2015

Dogdas B. et al. (2007). **Digimouse: a 3D whole body mouse atlas from CT and cryosection data.** *Physics in Medicine and Biology* (2007) 52, pp. 577-587

Edinburgh Mouse Atlas Project: http://www.emouseatlas.org/emap/ema/home.php - last check 8.9.2015

Fioroni, P. & Meister G. (1974). **Embryologie von Loligo vulgaris Lam. Gemeiner Kalmar.** *Gustav Fischer Verlag*, Stuttgart 1974, Großes Zoologische Praktikum Heft 16c/2

FishNet: http://www.fishnet.org.au/ - last check on 8.9.2015

Forbes J. G. et al. (2006) **Multimodal imaging of the sonic organ of *Porichthys notatus*, the singing midshipman fish.** *Magnetic Resonance Imaging* (2006), 24, pp. 321-331

Handschuh et al. (2010) **Showing their true colors: a practical approach to volume rendering from serial sections.** *BMC Developmental Biology* (2010), 10:41

Handschuh et al. (2013) **A correlative approach for combining microCT, light and transmission electron microscopy in a single 3D scenario.** *Frontiers in Zoology* (2013), 10:44

Hawrylycz M. et al. (2011). **Digital Atlasing and Standardization in the Mouse Brain.** *PLoS Computional Biology* (2011) 7(2): e1001065, doi:10.1371/journal.pcbi.1001065

Herdina A.N. et al. (2015) **Testing hypotheses of bat baculum function with 3D models derived from microCT.** *Journal of Anatomy*, 226(3), pp. 229-235

Jereb, P. & Roper, C. F. E. **Cephalopods of the World – an annotated and illustrated catalogue of cephalopod species known to date. Volume 1 – Chambered Nautiluses and Sepioids.** *Food and agriculture organization of the united nations*, Rome 2005, ISBN 92-5-105383-9

Kerbl A. (2012). **Development of the central nervous system in the Hawaiian bobtail squid (Euprymna scolopes) based on histology and x-ray microtomography.** Diplomarbeit, Universität Wien, 2012

Kerbl A. et al. (2013). **Micro-CT in cephalopod research: Investigating the internal anatomy of a sepiolid squid using a non-destructive technique with special focus on the ganglionic system.** *Journal of Experimental Marine Biology and Ecology* 447 (2013): pp 140–148

Kröger, B., Vinther, J. & Fuchs, D. (2011). **Cephalopod origin and evolution: A congruent picture emerging from fossils, development and molecules.** *Wiley periodicals Inc.* 2011, Bioessays 33: pp 602-613

Lee, P. N., P. Callaerts & H. G. de Couet (2009a). **Culture of Hawaiian bobtail squid (Euprymna scolopes) embryos and observation of normal development.** *Cold Spring Harbor Protocols* 2009(11): pdb prot5323.

Lee, P. N., P. Callaerts & H. G. de Couet (2009b). **The embryonic development of the Hawaiian bobtail squid (Euprymna scolopes).** *Cold Spring Harbor Protocols* 2009(11): pdb ip77.

Lee, P. N., M. J. McFall-Ngai, P. Callaerts & H. G. de Couet (2009c). **The Hawaiian Bobtail Squid (Euprymna scolopes): A Model to Study the Molecular Basis of Eukaryote-Prokaryote Mutualism and the Development and Evolution of Morphological Novelties in Cephalopods.** *Cold Spring Harbor Protocols* 2009, doi:10.1101/pdb.emo135

Louie A. Y. (2000) **In vivo visualization of gene expression using magnetic resonance imaging.** Nature Biotechnology (2000), 18, pp. 321-325

Mayer C. et al. (2014) **Studying developmental variation with geometric morphometric image analysis (GMIA).** *PLoS ONE*, 9(12), e115076

Metscher Brian D (2009a) **MicroCT for Developmental Biology: A Versatile Tool for High-Contrast 3D Imaging at Histological Resolutions**. *Developmental Dynamics*, 238:632-640

Metscher Brian D (2009b) **MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues.** *BMC Physiology*, 9:11

Mizutani R. and Suzuki Y. (2012) **X-ray microtomography in biology**. *Micron* (2012), Vol. 43, pp 104-115

morphdbase.de: https://www.morphdbase.de/ - last check on 8.9.2015

Moynihan M. (1983) **Notes on the Behavior of Euprymna scolopes (Cephalopoda: Sepiolidae)**. *Brill Behaviour* (1983), Vol. 85, No. ½, pp 25-41

Sands G. B. et al. (2005) **Automated Imaging of Extended Tissue Volumes Using Confocal Microscopy.** *Microscopy Research and Technique* (2005), 67, pp: 227-239

Sharpe J. (2004) **Optical Projection Tomography.** Annual Reviews Biomedical Engineering (2004), 6, pp 209-228, doi: 10.1146/annurev.bioeng.6.040803.140210

Shears J. (1988) **The use of a sand-coat in relation to feeding and diel activity in the sepiolid squid Euprymna scolopes**. *Malacologia* (1988), 29(1): pp 121-133

Storch V. & Welsch U. (2009) **Kükenthal – Zoologisches Praktikum**. Ausgabe 26, Spektrum akademischer Verlag (2009), ISBN: 3827419980

The Cephalopod Page: http://www.thecephalopodpage.org/Escolopes.php - last check on

8.9.2015

Wanninger A. (2007). **The application of microscopy and 3D imaging software in Functional, Evolutionary and Developmental Zoology: reconstructing myo- and neurogenesis in space and time.** *Modern Research and Educational Topics in Microscopy* (2007) pp. 353-361

Williams B. S. and Doyle M. D. (1996). **An Internet Atlas of Mouse Development.** *Computerized Medical Imaging and Graphics* (1996), Vol. 20, No. 6, pp. 433-447

Zebra fish atlas browser Univeristeit Leiden: http://bio-imaging.liacs.nl/atlasbrowserstart.html - last check on 8.9.2015

Ziegler A. et al. (2010) **Opportunities and challenges for digital morphology.** *Biology Direct* (2010), 5:45

Ziegler A. (2012) **Broad application of non-invasive imaging techniques to echinoids and other echinoderm taxa.** *Zoosymposia* (2012), 7, pp. 53-70

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

Lebenslauf

Persönliche Daten

Vorname:	Claudia
Nachname:	Klimpfinger
Wohnort:	Wien
Telefonnummer:	0699/ 10 51 00 16
E-Mail:	c.klimpfinger@gmx.at
Staatsbürgerschaft:	Österreich
Muttersprache:	Deutsch

Ausbildungsweg

Ab 2013: Masterstudium Zoologie
Universität Wien

2009 -2013: Bachelorstudium Biologie
Universität Wien

2004 – 2009: Handelsakademie
business.academy.donaustadt
Polgarstraße 24, 1220 Wien

2000 – 2004: Bilinguale Mittelschule (Englisch)
OMS Simonsgasse
Simonsgasse 23, 1220 Wien

Abschlussarbeiten und Publikationen:

Masterarbeit: „A 3D developmental atlas of *Euprymna scolopes* (Cephalopoda: *Sepiolidae*)“

Bachelorarbeit: „Die drei-dimensionale Rekonstruktion - mikroanatomischer Strukturen im Wandel der Zeit“
Universität Wien WS 2011/12

Allgemein

Sprachen:

- Deutsch in Sprache und Schrift (Muttersprache)
- Englisch in Sprache und Schrift, besonders flüssige Sprache durch bilinguale Schule und Sprachaufenthalte in England und Irland.
- Italienisch in Sprache und Schrift, zweimaliger Sprachurlaub in Rimini mit Besuch der Rimini Academy

Zusatzzertifikate:

Open Water Diver (Tauchschein)
Absolviert 2013
Führerschein der Klassen A und B
Absolviert 2009
Ausbildung zur Abfallbeauftragten
Absolviert im Schuljahr 2008/09
An der business.academy.donaustadt
Österreichisches Tanzleistungsabzeichen
Bronze, Silber, Gold, Goldstar, Platinstar
Sprachzertifikat der Rimini Academy
Corso Intensivo di Lingua Italiana
Absolviert von 16.11. – 27.11.2007
In Rimini, Italien
ATC Certificate English Language Studies
Upper Intermediate Course
Absolviert von 19.3. – 23.3.2007
In Bray, Irland
Zertifikat in Rechnungswesen und Controlling
Absolviert im Schuljahr 2006/07
An der business.academy.donaustadt
EF Language Travel Certificate
Language Course
Absolviert von 5.11. – 19.11.2003
In Eastborn, England

Berufserfahrung:

Studentenarchiv
Arvato Infoscore Austria GmbH
Juli 2011 – dato

Kassa
Wiener Bäder Gänsehäufel
Juli 2015

Hilfskraft Einkauf
CEMEX GmbH
Juli 2014

Hilfskraft Buchhaltung
WELDE GmbH
April 2010 – Februar 2011

Service
Konditorei AIDA
August 2010

Service
Ströck Gastronomiebetriebs GmbH
März-Mai 2010

Datenerfassung
AKH Wien
August 2009

Service und Verkauf
Müller & Gartner OHG
August 2007