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The “inner values” of an endoparasitic copepod – computer-based 3D-reconstruction of *Ismaila aliena*

(Copepoda, Poecilostomatoida, Splanchnotrophidae)

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Knowledge about the Splanchnotrophidae, a family of endoparasitic copepods infesting opisthobranch sea slugs, currently is restricted to the external morphology. In contrast, their internal anatomy is still largely unknown and many questions concerning life-history traits remain unanswered. Therefore, the microanatomy of both sexes of *Ismaila aliena* Haumayr & Schrödl, 2003, a splanchnotrophid infesting the nudibranch *Thecacera darwini* Pruvot-Fol, 1950 in Chile, was studied using computer-based 3D-reconstruction methods on serial semithin histological sections. The body musculature comprises three paired longitudinal strands. Regarding the cephalic and thoracic appendages, besides the antennae only the first pair of male thoracopods is supplied with strong musculature. The digestive system consists of an oesophagus and a voluminous, sack-like midgut, while hindgut and anus are lacking. Structural, functional and observational evidences suggest that *I. aliena* and at least some other splanchnotrophids are body fluid rather than – eponymous – tissue feeders. The gonad of *I. aliena* is large in both sexes and neither antrum nor seminal receptacle was detected in the female. Compared to ectoparasitic copepods, the central nervous system of *I. aliena* is modified, especially in males. Microanatomical results of the present study are compared with available literature results on *I. belciki* Ho, 1987 (as *I. monstrosa* Bergh) and discussed regarding potential functions. Within an emerging functional and evolutionary framework we provide some new insights in the life history of the splanchnotrophid parasites.

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Introduction

Copepods constitute one of the most important groups of marine zooplankton showing great diversity considering morphological characters (Yoshikoshi 1975). Especially parasitic copepods display diverse stages of adaptation, ranging from morphologically rather typical (ectoparasitic) forms like *Pararchinotodelphys gurneyi* Illg, 1955 to extremely specialised endoparasites like *Sacculina carcini* Thompson, 1836,

which is in its adult stage hardly recognisable as a crustacean. However, the internal anatomy of parasitic copepods is mostly unstudied except for species that have at least some economic relevance due to their respective hosts (Saby 1933, Østergaard 2004, Özel et al. 2004). Therefore members of the families Chondracanthidae and Lernanthropidae, parasites of commercially important fishes, were histologically analysed in detail in order to gain insight into their life history (Saby 1933, Rigby & Tunnell 1971, Clarke

& Klussmann-Kolb 2003, Molnár & Székely 2004). But for the majority of parasitic species causing no commercial damage comparable data are yet missing (Schrödl 1997, Özel et al. 2004). In the case of the small endoparasitic family Splanchnotrophidae, which specialises upon opisthobranch sea slugs, available morphological data are largely restricted to external characters (Huys 2001, Haumayr & Schrödl 2003, Anton & Schrödl 2013), which may be insufficient due to widespread organ reductions (Huys 2001). Our recent phylogenetic analysis of Splanchnotrophidae based on external morphological data provided a first testable phylogenetic hypothesis, but suffered from homoplasy, i. e. convergent developments reflecting the high level of adaptation in these endoparasitic species (Anton & Schrödl 2013). Histological studies may provide important additional data for a more detailed phylogenetic analysis and therefore advancing the understanding of copepod evolution.

In particular, there are some fundamental biological questions like nutrition or respiration, which cannot be answered by just looking at the external morphology (Clarke & Klussmann-Kolb 2003). Life-history traits of free-living or ectoparasitic copepods can be studied simply by cultivation under laboratory conditions or by analysing stomach contents (Gotto 1957, Nival & Nival 1976, Saiz & Kiorboe 1995, Wu et al. 2004). Unfortunately these methods seem rather unfitting for endoparasitic species. Although it is possible to see parasitizing females through the integument of the host in some splanchnotrophid species (Schrödl 1997, Abad et al. 2011), a detailed analysis especially of life-history traits from outside is impossible. Consequently, the mode of nutrition of the Splanchnotrophidae was discussed ever since the family was introduced. Hancock & Norman (1863) first suggested them to feed on inner organs and tissues of their hosts, which is reflected by how they named the type genus *Splanchnotrophus*. Huys (2001) also adopted this hypothesis of nutrition in his revision of the family. More recently, the absence of gnawing marks on the inner organs of the hosts led to the hypothesis that splanchnotrophids rather seem to be haemolymph suckers (Schrödl 2003, Anton & Schrödl 2013).

Rather than dissections the implementation of histological methods seems promising to gain reliable anatomical data from small-sized copepods. However, until today only one member of the family Splanchnotrophidae was subject of histological studies. Belcik (1965) examined the internal morphology of North-Eastern Pacific *Ismaila belciki* Ho, 1987 (as *I. monstrosa* Bergh), but despite bringing up interesting results further studies were never undertaken.

The present study analyses the internal anatomy of both sexes of *Ismaila aliena* Haumayr & Schrödl,

2003 (see Fig. 1) using histological semithin-sections of resin-embedded specimens. Computer-based microanatomical 3D-reconstruction techniques have been successfully applied on small molluscs such as sea slugs (e.g. Rückert et al. 2008, Brenzinger et al. 2013) and on arthropods (Brenneis & Richter 2010). Advantages of this method include better structural resolution, analytical scrutiny and efficiency to visualise the anatomy of highly complex organ systems of small specimens (Neusser et al. 2006, DaCosta et al. 2007). Exploring the internal anatomy and functions of both sexes of *Ismaila aliena*, chief purposes of the present study are to shed some light on the debated life history of the parasite, above all with respect to nutrition, respiration and mobility.

Material and methods

During a collection trip to Valdivia, southern Chile, in 2010 several infested nudibranchs were kept in the laboratory and observed for several days. Infested host specimens of the nudibranch sea slug *Thecacera darwini* Pruvot-Fol, 1950 were collected in the Bahía de Coliumo, central Chile in 2005. One male (ZSMA20130020) and one female (ZSMA20130021) of *I. aliena* were dissected from the 70 % ethanol preserved hosts. After removing the egg sacs they were dehydrated in an acetone series and were embedded in Spurr's low viscosity resin (Spurr 1969). Both specimens were serially sectioned (thickness 1.5 µm) following Ruthensteiner (2008) using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and sections were stained with methylene-azure II (Richardson et al. 1960). To obtain suitably sized sections, the tips of dorsal processes of the female were trimmed. Every section was photographed using a Jenoptik Prog Res C3 microscope camera (Jenoptik Laser, Optic, Systems GmbH, Jena, Germany) on a Leica DMB-RBE microscope (5×/0.15) (Leica Microsystems, Wetzlar, Germany). For the reconstruction of all major organ systems every section was photographed for the male and every second section for the female specimen. The computer-based 3D-reconstruction was performed with the software AMIRA 4.1 (TGS Europe, Mercury Computer Systems, Mérégnac, France) according to the protocol of Neusser et al. (2006) and Ruthensteiner (2008).

Results

An overview of the general external habitus is given in Figure 1. Ethanol-fixation allowed analysis of tissues rather than at cellular level.

Body

The body comprises of a cephalosome, bearing the head appendages and the mouthparts, the thorax

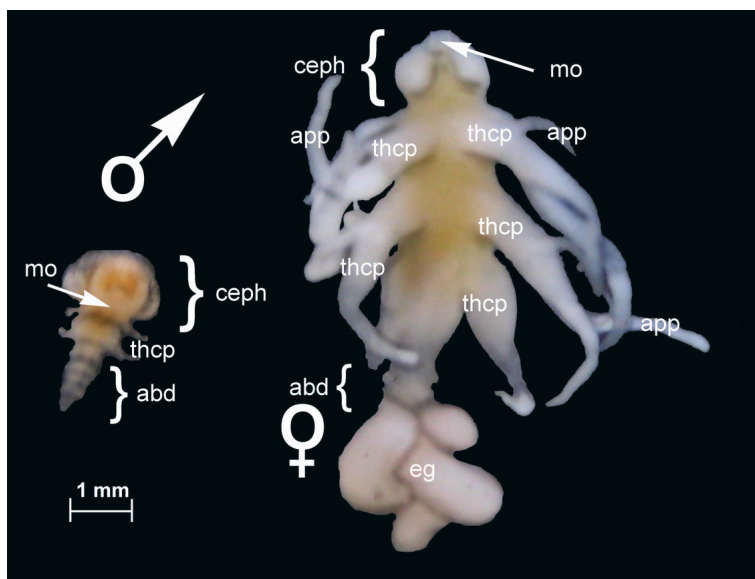


Fig. 1. General habitus of *I. aliena*. Ventral view of male and egg-bearing female. **abd**, abdomen; **app**, dorsal appendages; **ceph**, cephalosome; **eg**, egg sacs; **mo**, mouth; **thcp**, thoracopods.

bearing the modified thoracopods and the dorsal appendages in females, and of the short abdomen (Fig. 1). The body possesses three orifices: The mouth lying ventral in the head region and the paired genital openings lying laterally on the first abdominal segment. In both sexes no traces of an internal segmentation were detectable. The body wall shows an epidermis covered by a thin chitinous layer (see Figs 2 and 3).

Musculature

The female body shows three pairs of longitudinal muscles (see Fig. 4C). The dorsal strands lie close together and originate from the anterior part of the head. The strands of the ventral pair run further apart from each other, starting from the mouth area of the head. From these ventral muscles two lateral strands originate at the level of the third thoracic segment. All six resulting strands extend to the most posterior part of the abdomen (Fig. 4C), which can be retracted telescope-like in living animals. Concerning the mouthparts, the antennae are equipped with the strongest muscles. Mandibles and maxillae only possess thin strands of musculature. Further two strands of musculature, originating directly from the starting point of the ventral longitudinal muscles, run transversely along the ventral border of the head region (see Fig. 4C). At the level of the second thoracic segment a pair of V-shaped muscles reaches from the dorsal side of the body into the

first segment of the thoracopods (Fig. 4C). Neither in the thoracopods nor in the dorsal appendages any musculature could be detected.

In the male, two pairs of longitudinal muscles – e.g. the lateral and the ventral pairs – reach from the mouth region of the cephalosome to the posterior end of the abdomen (see Fig. 5C). The lateral and the ventral strand are connected by a transverse strand of muscle at the level between the first and second pair of thoracopods. Transverse and longitudinal strands are directly connected in areas attached to the cuticle. An additional dorsal pair of longitudinal muscle strands reaches from the frontal area of the cephalosome to the level of the first thoracic segment and is divided into four parts by connections to the body wall (Fig. 5C). The antennae and the first pair of thoracopods show strong musculature. In the first two pairs of thoracopods each first segment is equipped with a single muscle strand reaching to the lateral sides of the respective segment (Fig. 5C).

Digestive system

The female digestive system comprises a mouth and a short oesophagus followed by a voluminous tube-like midgut (see Fig. 4D). The midgut reaches from the head region to the level of the third pair of dorsal appendages (Fig. 4). The walls of the midgut are straight and show no lumen-sacs as mentioned by Saby (1933) for *Parabrachiella insidiosa* (Heller, 1865). In the whole lumen of the midgut, gland cells

as described by Saby (1933) can be found either isolated and free in the lumen or attached to the inner wall of the midgut (Figs 2 and 3). In the female, the gland cells are more or less homogeneously dispersed (Fig. 2), while in males they are aggregated at the anterior and posterior end of the midgut and in the area around the mouth (Fig. 3). In females, a small sac-like structure is present on the right side at the posterior end of the midgut (see Fig. 4D). Additional digestive organs (e.g. gut or rectum) or an anal opening are absent.

The digestive system of the male largely resembles that of the female; the large, triangle-shaped sack-like midgut also fills great parts of the body cavity but reaches only to the level of the second pair of thoracopods (see Fig. 5D). No traces of either hindgut or anal opening were detectable.

Reproductive system

The reproductive system of the female consists of an unpaired ovary, paired oviducts and an unpaired cement-gland (Fig. 4B). The ovary stretches through the whole body – including dorsal processes and thoracopods – and sends branches even into the head region (cephalosome). At the level of the third thoracic segment the ovary is connected to the paired oviducts by thin and short ducts (Fig. 2C). The voluminous oviducts lead to the genital openings located on the ventro-lateral sides of the first abdominal segment. Dorsally, close to the midgut, an unpaired cement-gland is present which tapers into one single duct reaching to the beginning of the abdomen; there, it separates into two ducts, which are enlarged at the level of the last thoracic segment (see Fig. 4B). Although the ducts run partly alongside the oviducts, they do not fuse with them until reaching the genital openings on the first abdominal segment. A receptaculum seminis or an antrum as described by Schminke (2007) could not be detected.

In the male, the paired testes are located dorsally in the cephalosome, filling its lumen to the greatest part (see Fig. 5B). The paired vasa deferentia are long and entwined. They are enclosed by strong musculature and lead to paired seminal vesicles, where the spermatophores are formed (as described by Schram 1986) and which are connected to the genital openings in the first abdominal segment (Fig. 5B).

Nervous system

The nervous system of the female consists of a supra-oesophageal ganglion and an elongated infra-oesophageal ganglion tapering into a ventral nerve cord (see Fig. 6A). Both ganglia are connected

by two massive circum-oesophageal connectives. The ventral nerve cord appears unpaired with five small ganglia and terminates at the level of the first thoracic segment (Fig. 6A). The organisation of the nervous system shows the highest level of variation between male and female *I. aliena* apart from the gonads. The nervous system of the male consists of a circum-oesophageal nerve ring (gullet-ring), and a supra-oesophageal ganglion, which is drawn out ventrally (see Fig. 6B). A ventral nerve cord like in the female could not be detected. In both sexes the supra-oesophageal ganglion seems to represent the largest part of the brain (Fig. 6). A nauplius eye was neither found in the female nor in the male. The reconstruction of the nervous system is limited to the most conspicuous parts, since for a detailed reconstruction especially of the branching nerves other methods (e.g. Bundy & Paffenhöfer 1993, Geiselbrecht & Melzer 2013) are necessary. Hence the terminology of the distinct parts refers to Saby (1933) since for the identification of proto-, deutero- and tritocerebrum (Lowe 1935) the exact innervation of all cephalic and thoracic appendages is required.

Excretion and circulation

In both sexes, a rather small, paired structure was found on the ventral sides of the cephalosome, which we assume to represent the antennal glands (see Figs 4D, 5D and 6). Neither a heart or other circulatory organs nor any special respiratory organs could be detected.

Discussion

One of the few authors dealing with the internal anatomy of parasitic copepods is Saby (1933) who examined and described six species of the ectoparasitic families Chondracanthidae and Lernaepodidae in great histological detail. Belcik was the first to study the internal anatomy of members of the Splanchnotrophidae, i.e. describing both sexes of *I. belciki* (as *I. monstrosa*) in his doctoral thesis (Belcik 1965) and publishing on the male later (Belcik 1981). Although providing many new insights, such studies were limited by the paraffin-based methodology used at those times. Both specimens available for examination herein were fixed in 70 % ethanol; preservation was good enough to distinguish tissues and recognise organs. Here we compare and supplement the initial data provided by Belcik (1965, 1981) using modern semi-thin histological and serial 3D microanatomical reconstruction techniques for the first time for splanchnotrophids.

Body wall

In *I. aliena* the chitinous outer layer of the body wall is thin throughout the whole body, which was also mentioned for *I. belciki* by Belcik (1965, 1981); therefore the parasite appears soft and fragile during macroscopic preparation. Papillae or other structures related to respiration could not be detected. This indicates that the entire body surface is involved in exchange of gases, confirming earlier assumptions (Salmen et al. 2008b, Anton & Schrödl 2013). The body wall is slightly thickened only in the area around the mouth and at the mouthparts. In addition, the distal part of the abdomen, which becomes ingrown in the body wall of the host or is in contact with the seawater outside of the host, is equipped with a very strong chitinous layer and a thick epithelium, obviously for stability and protective reasons.

Body musculature

The musculature found in *I. aliena* greatly resembles *I. belciki*, but Belcik (1965, 1981) mention only four strands of longitudinal muscles. However, in his drawings six strands of longitudinal muscles can be seen (Belcik 1965 figs 13B and 14A) so he may simply not have counted the lateral ones as independent strands. In contrast, the musculature of *I. aliena* shows considerable differences compared to the results of Saby (1933). For example in *I. aliena* longitudinal and cephalic muscles are not originating from a ring of strong musculature and all species examined by Saby (1933) are equipped with only four strands of longitudinal muscles.

Female *I. aliena* have additional muscles running transverse from the starting point of the ventral longitudinal muscles to the ventral side of the cephalosome. Neither Saby (1933) nor Belcik (1965) mentioned any such muscles and their function remains unclear. The strong longitudinal musculature present in *I. aliena* seems to serve particularly to retract the parasite's abdomen. This behaviour can easily be observed in egg-bearing females by touching them with a forceps. The female will retract its abdomen and create a fold in the integument of the host to protect the eggs (personal observation). The antagonist of this retraction may be the internal body pressure of the parasite on the one hand and on the other hand the elastic mantle tissue of the host. Both sexes need to extend their abdomen through the hosts' body tissue and telescope-like extension may help with penetrating; then their abdomen, with special abdominal ring in *Ismaila* (see Anton & Schrödl 2013), is firmly embedded in the host tissue.

Male *I. aliena* are equipped with stronger longitudinal musculature than females. One reason could

be that in *I. aliena* the abdomen of anchored males is not in direct contact with that of the female. Therefore the retraction of the abdomen is possibly needed to make contact between the male and female genital porus. An additional explanation refers to the assumed higher mobility of male Splanchnotrophidae (Ho 1987, Schrödl 1997, Anton & Schrödl 2013).

Musculature of the thoracopods

Following the drawings provided by Saby (1933), the v-shaped muscles found in female *I. aliena* represent the only remnants of the thoracopod musculature (Fig. 4C). According to muscle arrangement, females seem to be able to move the first pair of thoracopods only as a whole, i.e. we could not detect any significant musculature indicating that the thoracopods are able to perform complex movements like grasping.

It has been assumed that in parasitic copepods males often represent the lesser-modified state of development (Saby 1933, Huys 2001). Considering the strong thoracopodal musculature of *I. aliena* (Fig. 5C), this hypothesis is supported. Possible reasons for this sexual dimorphism refer to locomotion and mating behaviour and will be discussed below.

Locomotion

The only musculature remaining for the purpose of locomotion in female *I. aliena* are the longitudinal muscles. The different strands are contracted alternately in the female resulting in a kind of movement similar to nematode worms such as *Ascaris* (personal observation). During this movement, the strong antennae may be used for grasping so that the rest of the body can be pulled forward; however, female *I. aliena* are no longer capable of any efficient directional movements (Schrödl 1997, personal observation).

The high degree of adaptation to semi-sessile endoparasitic life is revealed most obviously when observing the efforts made by a female parasite leaving its host (Schrödl 1997). Splanchnotrophid parasites may try to escape, if their host is in bad physical condition (e.g. injured or undernourished). In female *Ismaila* this escaping-behaviour is restricted to opening the host's integument and crawling out of the body cavity but the abdomen will remain embedded in host tissue (Schrödl 1997, personal observation). The reason for this behaviour is still unknown since both individuals (parasite and host) will die shortly after the parasite emerges from its host (Schrödl 1997, Abad et al. 2011). Males, however, are capable of freeing their abdomen. There is little chance to find and infest a new host, but male *Ismaila*

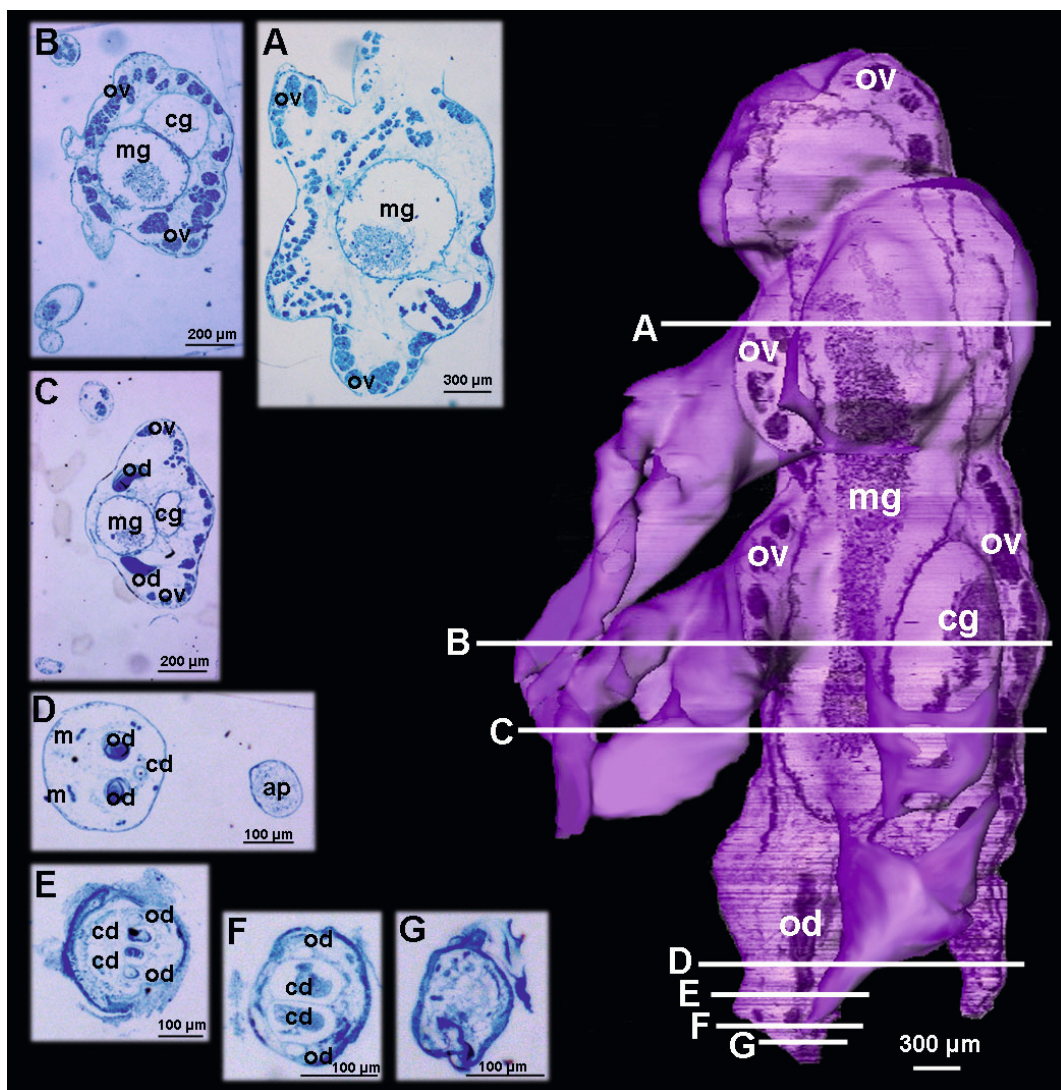


Fig. 2. Lateral view of the female with the indication of the inner organs as they can be seen on a medial section (digital reslice). Bars indicate the levels of the particular original sections A to G. ap, medio-dorsal appendage; cd, cement gland duct; cg, cement gland; m, musculature; mg, midgut; od, oviduct; ov, ovary.

may be able to leave a certain position at the body wall, move inside their host and mate with other females.

The higher complexity and stronger body musculature in males (Fig. 5C) suggests that mature males retain a higher degree of mobility than females from larval stages which we observed migrating freely inside the body cavity of the host. Males often are found “swimming freely” in the body cavity of the host (Ho 1981, 1987, Haumayr & Schrödl 2003, Abad et al. 2011), and need to get in touch with a female

for copulation. The body cavity of nudibranchs is not very spacious but rather tightly packed with inner organs (e.g. Martynov et al. 2011), moving larvae and males thus may rather “crawl” inside the body cavity than actually “swim”, e.g. by using the antennae and the first pair of thoracopods. We also observed such specimens of *I. aliena* penetrating the body wall of the host quite quickly, exiting (but not really leaving the host) with cephalosome first (Schrödl 1997). There should be a way to cut or destroy host tissue with the head.

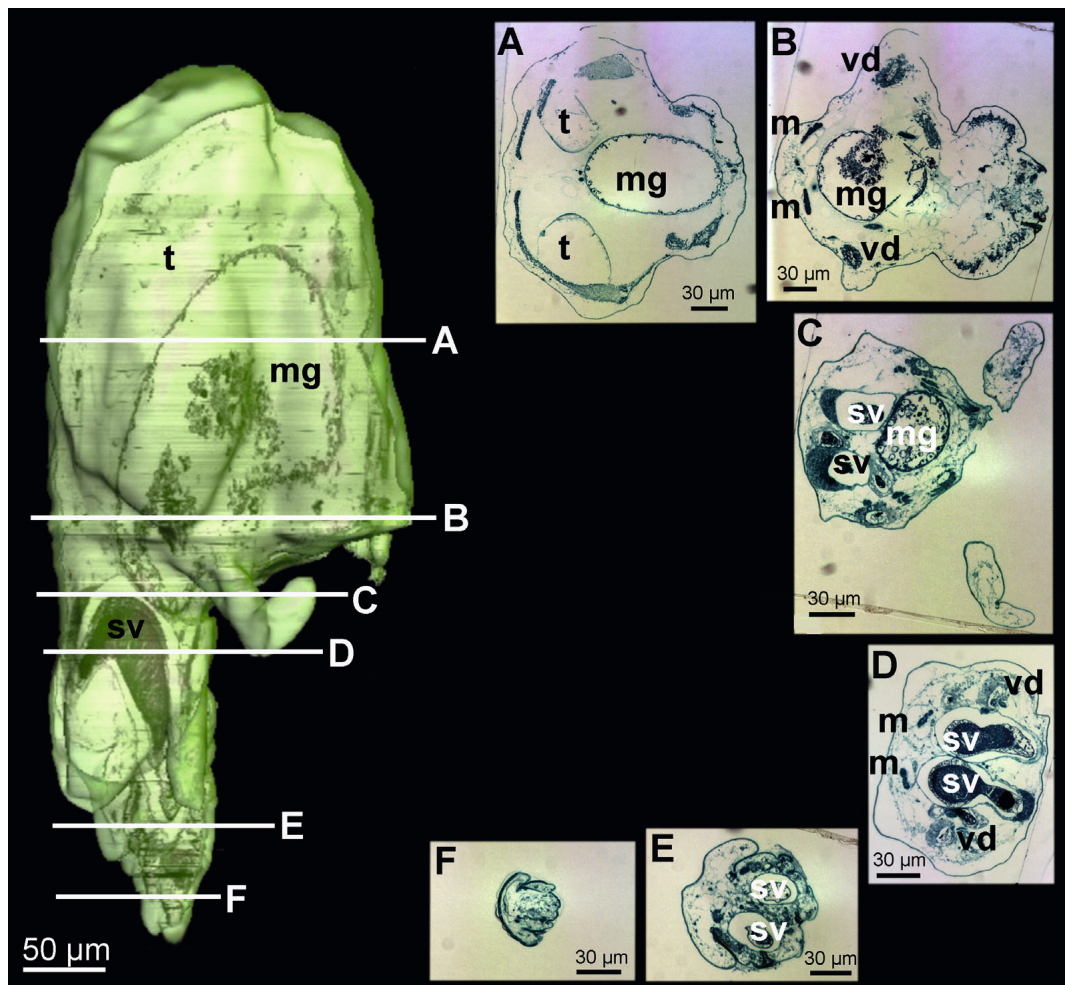


Fig. 3. Lateral view of the male with the indication of the inner organs as they can be seen on a medial section (digital reslice). Bars indicate the levels of the particular original sections A to F. m, musculature; mg, midgut; sv, seminal vesicle; t, testes; vd, vas deferens.

Cephalic muscles

In *I. aliena* no musculature could be detected in the antennulae, which is consistent with the assumed function of a sensoric device (Schram 1986). In ectoparasitic species the antennae are mainly used for attachment to the host (Schram 1986, Boxshall 2005) and therefore it is most interesting that the antennae are still present in endoparasitic Splanchnotrophidae, even in genera like *Arthurius*, where the mouthparts are already partially reduced. Hence the antennae still seem to have an important function. In fact, the strongest musculature found in *I. aliena* concerning cephalic appendages serves the claw-like antennae (Figs 4C and 5C). We thus assume that antennae are

used as a device for anchoring the parasite during movements or copulation. Antennae may have further functions, such as destroying host tissue during migration and for perforating the body wall of the host. Although most of splanchnotrophid species do not harm their hosts, Bergh (1867), Jensen (1987) and Schrödl (1997) described the gonads of host individuals of *Ismaila monstrosa* Bergh, 1867, *I. jenseniana* Haumayr & Schrödl, 2003 and *I. dammosa* Haumayr & Schrödl, 2003 as partly destroyed or damaged. All three authors did not assume this to be for the purpose of feeding, but rather to gain space. We conclude that in these cases the antennae are used to dissect and remove the particular organ.

All species examined by Saby (1933) are blood-sucking fish parasites located on the gills of their respective host. However, all these species still have strong muscles serving the mouthparts and the oesophagus (Saby 1933) to rasp off tissue and gain access to blood vessels. Even species like *Brachiella obesa* (Krøyer, 1837) and *Clavella uncinata* (Müller O. F., 1776) (synonym of *Clavella adunca* (Strøm, 1762)), which show strong modifications of the head, still have strong muscles around the oesophagus (Saby 1933). Such musculature is missing in *I. aliena*, contradicting a similar tissue-feeding mode.

In the tiny, sickle-shaped mandible of *I. aliena* no strong muscle strands were detectable, whereas in the maxillulae and the maxillae several strands of musculature were found. This supports the hypothesis that the latter two pairs of mouthparts rather than mandibles play an active role during feeding in *Ismaila*.

Digestive system and feeding mode

In his study on *I. belciki*, Belcik (1965, 1981) describes the digestive system as incomplete since no intestine, rectum or anal opening was evident. The present analysis on both sexes of *I. aliena* confirms the absence of any hindgut or anal opening in *Ismaila* species. Several histological studies on tissue-feeding ectoparasitic copepods showed that their digestive systems are complete, i.e. form a flow-through system with separate intestine, which is the general copepod pattern (Najarian 1952, Hartmann 1986). In contrast, parasitic copepods with females showing an incomplete digestive system are known to feed on body-fluids – mostly haemolymph – of their invertebrate hosts (Gotto 1979 and references cited therein). We conclude that finding of a blind-ending digestive system in both sexes of *I. aliena* and *I. belciki* also indicates feeding on fluids rather than tissue. This supports our hypothesis that at least some splanchnotrophids feed on haemolymph (Schrödl 2003, Anton & Schrödl 2013).

In his revision of copepods associated with marine invertebrates, Gotto (1979) mentions three species lacking any sign of an alimentary canal. Since the chitinous layer in all these copepods was found to be very thin, the uptake of nutrients by absorption was the accepted explanation in all three cases (Paterson 1958, Bresciani & Lützen 1960, Vader 1970, Gotto 1979). *Ismaila aliena* has a thin body cuticle and a midgut; thus some nutritional uptake through their thin body cuticle as suggested by O'Donoghue (1924) is possible but an additional rather than main food source.

In the case of fish-parasitizing, blood feeding *Chondracanthus lophii* Johnston, 1836, only the male

possesses an incomplete digestive system with a midgut ending as a blind sac (Østergaard 2004). Østergaard (2004) assumed that those males feed on special secretions produced in the glands of the female nuptial organ. In contrast, the digestive system of both sexes of *I. aliena* is quite similar in structure, suggesting a rather similar mode of nutrition. Furthermore the remaining parts of the digestive system in *I. aliena* show no signs of enhanced functionality. The sac-like midgut has straight walls instead of foldings or eversions like the lumen sacs described by Saby (1933) to maximise the surface (Figs 2 and 3). This indicates that the food of *I. aliena* is nutrient rich and rather easy to digest.

Observations on a female individual of *Ismaila* sp. found infesting a living though heavily distorted host, i.e. an aeolid nudibranch *Flabellina* species (*Flabellina* sp. 1 according to Schrödl 2003) collected in Chile in 2010 may shed further light on the question of nutrition of Splanchnotrophidae. The female parasite was enclosed by a sack-like evagination of the host's mantle tissue, having only one narrow tube-like connection to the body cavity and therefore to the haemolymph of the host (see Fig. 7), but not to any visceral organs. This very unusual position of the parasite may have resulted from the smallness of the host individual into which the fully-grown female parasite simply would not fit. Since access to other food sources than haemolymph probably has ceased earlier, and the parasite appears fully functional, i.e. reached maturity and even developed egg sacs; the only possible resource of nutrition is the haemolymph.

Accepting that *Ismaila* feeds on haemolymph of sea slugs – a fluid with dissolved nutrients and some cellular contents – explains why important parasite structures like parts of the gut and digestive glands could be reduced. Brooker et al. (2007) pointed out that digestion in blood-feeding parasites is slow but complete; however, indigestible residues also could be disgorged through the mouth or embedded in the parasite's body in form of crystals, as has been found for blood-feeding parasites of other taxa (Perkins 1985, Boxshall 2005). In the case of *I. aliena* no traces of such crystals could be detected, but they might have been dissolved and/or lost during the embedding process. In *I. aliena* a small structure was detected at the posterior end of the midgut which, according to the drawing provided by Schram (1986), could be an oil sack (see Fig. 4D). Its poor development may reflect the endoparasitic life of splanchnotrophids, i.e. living in an environment of constant food supply they are not as dependent on stored nutrients as for example free-living predatory species.

Feeding on a fluid is consistent with the reduction of mouthparts in some genera of the Splanchn-

notrophidae, such as *Arthurius* Huys, 2001 and *Ceratosomicola* Huys, 2001 (see Huys 2001, Salmen et al. 2008a,b). Anton & Schrödl (2013) assume that splanchnotrophid antennulae and claw-like antennae rather than mouthparts are involved into larval host detection and penetration. Indeed, the mandible of splanchnotrophids is rather inconspicuous, but maxillae and maxillules can be well developed. Inferring their function one has to consider that according to Schminke (2007), the viscosity of haemolymph is, for small animals like copepods, more similar to thick honey than to water. In such an environment the brush-like maxillae found in all *Ismaila* species (Hau-mayr & Schrödl 2003) would rather function as some sort of spoon to shovel the viscous haemolymph into the mouth and hereby fill the midgut. After the absorption of nutrients compression of the midgut through a contraction of the whole body, i.e. by the longitudinal muscle strands, could simply disgorge its content. The only conceivable alternative to suck in the haemolymph would require strong strands of musculature surrounding both oesophagus and midgut (Kaestner 1967). But no traces of such muscles could be found in either sex of *I. aliena* (Figs 2 and 3) and Belcik (1965) also mentions no such musculature in his study of *I. belciki*.

In her study on several members of the Splanchnotrophidae using scanning electron microscopy Salmen (2005) indicates the presence of an anal opening in *Splanchnotrophus angulatus* Hecht, 1893, *S. gracilis* Hancock & Norman, 1863 and *Ceratosomicola mam-millata* Salmen, Wilson & Schrödl, 2008. Whether this means that the nutrition of these species differs from that described herein yet needs to be clarified. The same uncertainty applies to *Arthurius*, since in this genus all mouthparts except the maxillae are reduced. At first glance reduction of mouthparts seems to be consistent with our hypothesis of fluid rather than tissue feeding, but in *Arthurius* the maxillae are claw-like (Salmen 2008a) and do not have the brush-like appearance described above. Therefore an alternative method of feeding may be possible. Concluding, there is still need of further investigation to finally resolve the exact mechanisms of feeding across members of the family Splanchnotrophidae, with genera showing considerable variation regarding mouthparts and possibly digestive systems.

Female reproductive anatomy

In female *I. aliena* there are two bulbous structures, which may be determined as paired ovaries, as is typical for parasitic copepods (Kaestner 1967, Schminke 2007). But according to the drawings provided by Saby (1933), the ovaries are distinctly set off from the oviduct. However, in *I. aliena* there is no

clear distinction between these bulbs and the highly branched structures traversing the whole body of the female, which therefore are also determined as parts of the ovaries. Since the ovaries merge at several locations the entire structure may have derived from a secondary fusion of once paired ovaries. These anastomosing ovaries occupy the greatest part of the entire body lumen, reaching as far as into the dorsal appendages and even the thoracopods (see Fig. 4B), optimising ovary volume and surface in a bizarrely shaped endoparasite.

In *I. aliena* the ovaries connect to a pair of oviducts. The oviducts and the cement-gland duct stay separate until the genital opening (Fig. 4B). Since in addition no receptaculum seminis could be detected, an antrum as described by Schminke (2007) must be considered absent. The ectoparasitic copepods described by Saby (1933) and *I. belciki* studied by Belcik (1965) also lacked an antrum, but all these species still showed a receptaculum seminis. The most striking difference between *I. aliena* and the ectoparasites studied by Saby (1933) is the morphology of the cement-gland, which produces the egg sacs. While in the ectoparasitic species a pair of lateral cement-glands is always present, the cement-gland of *I. aliena* is unpaired and lies dorsal to the midgut (Fig. 4B). Interestingly not only glands are fused in *I. aliena*, but also the cement-gland ducts at least until reaching the genital segment (see Fig. 4B). In the histological sections a structure is visible inside the cement-gland duct, which is also separating at the level of the genital segment and follows the ducts until the genital openings (Fig. 2). Probably this structure represents the tissue secreting the envelope of the egg sacs.

Belcik's (1965) description of the female reproductive system differs from the present one regarding the nomenclature of its distinct parts. He described the reproductive system of *I. belciki* as consisting of paired ovaries, paired but fused oviducts, paired cement-glands and an unpaired receptaculum seminis (Belcik 1965). Although the general morphology of both reproductive systems shows great similarities, we tend to a different interpretation. Belcik (1965) assumed that the oviduct is leading through the paired cement-glands. But in the histological section of *I. aliena* it is clearly visible that there is no trace of glandular tissue enwrapping the oviduct (Fig. 2). On the contrary, the structures as a whole have a connection to the highly branched ovary (see Fig. 2C); therefore, and according to the appearance of its content (see Fig. 2), they should be regarded as paired oviducts.

The results of the present study also favour a different interpretation of what Belcik (1965) assumed to be an unpaired receptaculum seminis. First of

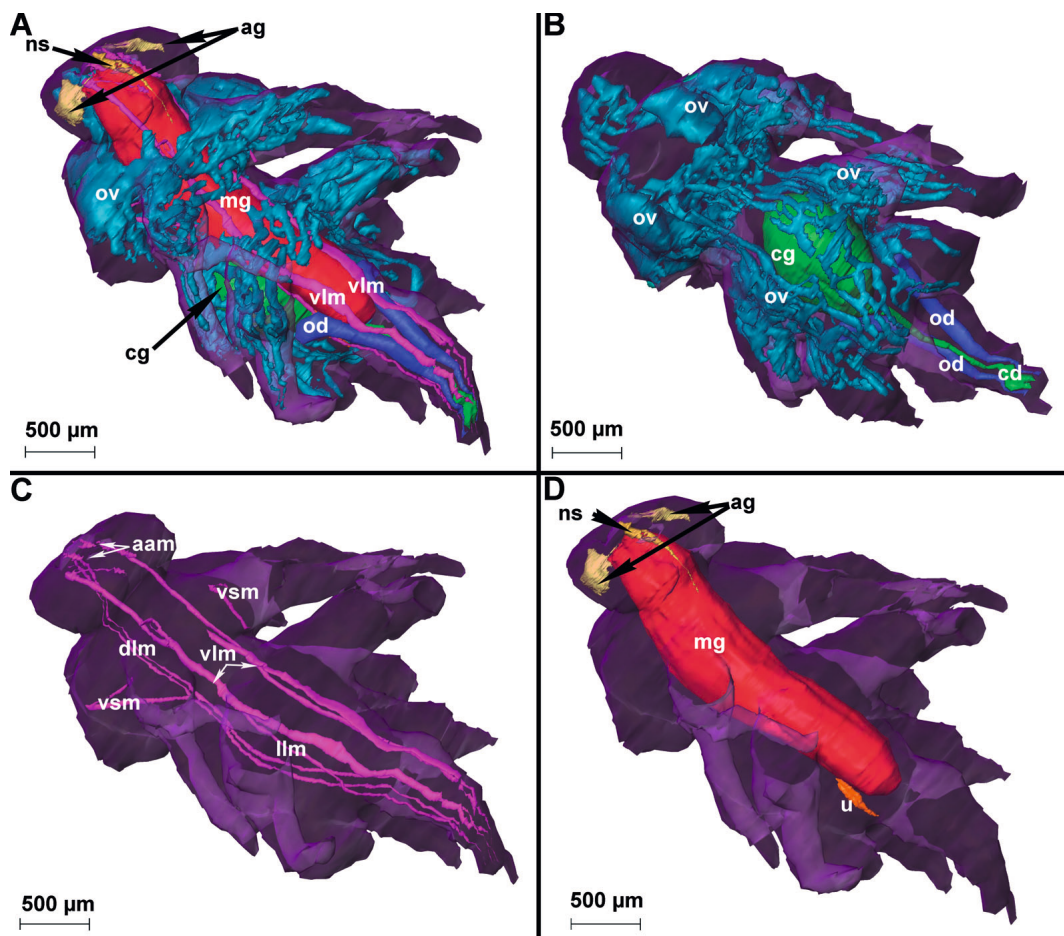


Fig. 4. 3D-reconstructed model of the internal anatomy of female *I. aliena* (ventro-lateral view). **A.** Overview of the complete internal morphology; **B.** reproductive system (dorsal view); **C.** musculature; **D.** digestive system, nervous system and excretory glands. **aam**, antennal muscles; **ag**, antennal gland; **cd**, cement gland duct; **cg**, cement gland; **dlm**, dorsal longitudinal muscles; **llm**, lateral longitudinal muscles; **mg**, midgut; **ns**, nervous system; **od**, oviduct; **ov**, ovary; **u**, unidentified structure (potential oilsac); **vlm**, ventral longitudinal muscles; **vsm**, v-shaped muscles.

all the content found inside the relevant structure looks different compared to the one found inside the seminal vesicles of the male and, therefore, do not seem to represent male gametes or spermatophores (see Figs. 2 and 3). In addition, the posterior region of the duct seems to contain a glandular structure possibly to synthesise the material of the egg sacs (Fig. 2), and this structure seems to be rather voluminous compared to the relative body size. Even in ectoparasitic species where males are not present all the time the receptaculum seminis usually is a tiny structure (Saby 1933, Najarian 1952). Considering that in *I. aliena* males and therefore male gametes are constantly available for the female makes the presence of a receptaculum seminis of this size rather

unlikely. In summary, we assume that this structure rather represents an unpaired cement-gland than a receptaculum seminis.

Male reproductive anatomy

In male *I. aliena* the paired testes are located in the posterior region of the cephalosome, filling it to the biggest part (see Fig. 5B). In copepods the testes usually are situated in a similar position (Kaestner 1967, Schminke 2007), but are rather small, sometimes even unpaired and the vasa deferentia provide a rather straight connection to the seminal vesicle where the spermatophores are produced (Saby 1933, Schminke 2007). In his study on male *I. belciki*, Belcik (1981)

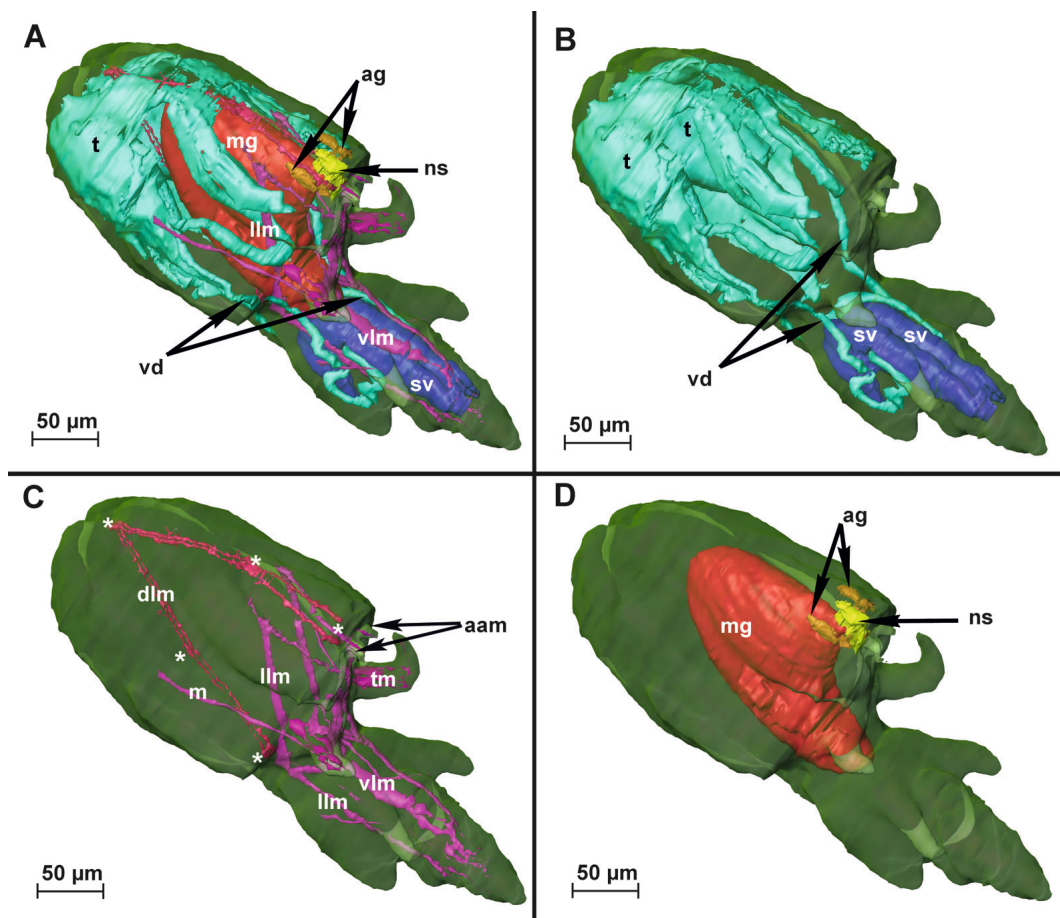


Fig. 5. 3D-reconstructed model of the internal anatomy of male *I. aliena* (ventro-lateral view). **A.** Overview of the complete internal morphology; **B.** reproductive system; **C.** musculature; **D.** digestive system, nervous system and excretory glands. **aam**, antennal muscles; **ag**, antennal gland; **dlm**, dorsal longitudinal muscles; **llm**, lateral longitudinal muscles; **m**, muscle strand connected to the thoracopods; **mg**, midgut; **ns**, nervous system; **sv**, seminal vesicle; **t**, testes; **tm**, internal thoracopodal muscles; **vd**, vas deferens; **vlm**, ventral longitudinal muscles. Asterisks indicate the areas where the dorsal longitudinal muscles are connected to the body wall, thus forming four parts.

already noted that the testes extend dorsolaterally into the swollen segments of the cephalothorax, with the vasa deferentia running along them laterally in an uneven or convoluted manner (Belcik 1965, 1981). We found that a layer of strong muscles enwraps the meandering vasa deferentia in *I. aliena*, possibly to transport the gametes from the testes to the seminal vesicles by peristaltic movement (see Fig. 3).

Mating biology

The act of copulation still is completely unknown for splanchnotrophids and therefore it is possible that sperm transfer takes place before the female

anchored its abdomen in the hosts' integument. In that case males would be expected to search for migrating females rather than joining already anchored females. However, in nearly all splanchnotrophid species males were found anchored close to females (Ho 1981, Schrödl 1997, Huys 2001, Haumayr & Schrödl 2003, Salmen et al. 2008b, Abad et al. 2011). We thus assume that copulation takes place continuously between males and females anchored close together and therefore a constant supply of male gametes is provided. In the genus *Ismaila* the female is usually flanked by two or three anchored males (Haumayr & Schrödl 2003) in a way that the genital opening of the males are near those of the female,

but males also are found freely inside the host (Ho 1987, Haumayr & Schrödl 2003). According to the arrangement of anchored males and females, the offspring of one female may have different fathers, depending on whether they hatch from the left or the right egg sac. This would be remarkable, since usually copepod males are anxious to ensure to be the only one the female copulates with and many free-living species therefore show mate-guarding behaviour (Anstenrud 1992, Todd et al. 2005, Titelman et al. 2007). Further studies are necessary to confirm and explain the variable number of males aggregated to females in different species (Schrödl 1997, Huys 2001, Haumayr & Schrödl 2003, Salmen et al. 2008a,b, Abad et al. 2011) and to explore the genetic diversity of splanchnotrophids.

Circulation

As it is typical for cyclopoid copepods (Kaestner 1967, Schram 1986, Schminke 2007), a heart or other circulatory organs are missing in both sexes of *I. aliena*. It is assumed that the movement of the body itself maintains the circulation of the haemolymph (Saby 1933, Kaestner 1967, Schram 1986, Schminke 2007). This could either be by passive movement, which is induced by the movements of the host, or actively by the parasite itself. Indeed it has been observed that the retraction of the abdomen is performed by female *I. aliena* without any visible tactile stimulus (personal observation).

Excretory glands

In both sexes of *I. aliena* a paired structure in the head region was detected (see Figs 4D, 5D and 6), which is assumed to represent a pair of antennal glands as described by Schram (1986), since their ducts are leading outwards at the level of the antennae. This interpretation needs reconfirmation, however, since usually a pair of maxillary glands maintains the excretion in copepods (Claus 1880, Saby 1933, Kaestner 1967, Schminke 2007). In *I. aliena* the size of these antennal glands in relation to the body size is similar in both sexes, which is in accordance with the major function of these glands being excretion of metabolic waste. Neither frontal glands nor maxillipedal glands, structures that are otherwise indicated to be involved in excretion as described by Saby (1933), could be found in *I. aliena*.

Nervous system and sensory functions

The nomenclature of the nervous system refers to Saby (1933). For the identification of proto-, deutero- and tritocerebrum (Lowe 1935) the exact innervation

of all cephalic and thoracic appendages is required, which we could not resolve in our specimens due to inadequate fixation. The nervous system of *I. aliena* could be reconstructed in both sexes, and it shows a significant sexual dimorphism. This is quite remarkable, since according to Saby (1933) and Weatherby et al. (2000), the nervous systems of male and female copepods are usually rather similar. In *I. aliena* the supra-oesophageal ganglion appears to be the largest part of the nervous system (Fig. 6A). In males it is not only enlarged but also distinctly set off from the circum-oesophageal nerve ring. Such structural differences may refer to different functions that are relevant to male and female *Ismaila*.

In general the nervous system of cyclopoid copepods consists of a supra-oesophageal ganglion, strong connectives encircling the oesophagus and a ventral nerve cord, which reaches to the end of the thorax (Saby 1933, Schram 1986). The supra-oesophageal ganglion mainly innervates the antennulae and antennae (Saby 1933, Schram 1986). The antennulae are assumed to function as major sensoric devices (Schram 1986) in copepods. Especially in splanchnotrophid copepods antennulae are assumed to play an important role during locating and identifying suitable hosts in the infective copepodite I stage (Ho 1987). In this context it is interesting that only two conditions have been observed in splanchnotrophids: either one or more female(s), or male(s) and female(s) (Huys 2001, Schrödl 2002, Haumayr & Schrödl 2003, Marshall & Hayward 2006, Abad et al. 2011). This would imply that either male copepodite I exclusively are attracted by or infest hosts already bearing a female, or that in the copepodite I state the sexual determination is not yet permanent and the first larvae entering a new host always develops into a female. Antennulae thus could play a role in male determination in an already infested host. Even a facultative sex reversal as described by Dharami & Altaff (2002) and Fleminger (1985) could be possible; unfortunately the distinct mechanisms of infection or sex determination are yet unrecognised (Ho 1987, Schrödl 1997, Anton & Schrödl 2013). Migratory larval stages and premature males and females may use antennulae for orientation in the host and monitoring the chemical environment, and also trigger the escaping behaviour of the parasites discussed above.

In adult males the antennulae may be used to find a mate. In nearly all splanchnotrophid species there have been reports of adult males lying freely in the body cavity (Ho 1987, Huys 2001, Haumayr & Schrödl 2003, Abad et al. 2011), which need to detect a female, then move towards it and anchor its abdomen nearby. However, positions and time scales of larval maturation in the hosts are still unclear.

In female *I. aliena* the supra-oesophageal ganglion is connected to the infra-oesophageal ganglion through strong connectives. In general the infra-oesophageal ganglion innervates the mouthparts (Schram 1986). According to our fluid-feeding hypothesis discussed above, in *I. aliena* this should mainly be maxillulae and maxillae. In the male the infra-oesophageal ganglion and the connectives are fused to a circum-oesophageal nerve ring. There are no obvious differences between female and male mouthparts, thus the reasons for this fusion remain unclear.

In the female a ventral nerve cord with five distinct bulbs – possibly ganglia – could be detected (see Fig. 6A). According to Schram (1986) the ventral nerve cord mainly serves the thoracopods and the abdomen but usually shows no distinct ganglia. Since none of the female thoracopods bears any strong musculature the purpose of these five ganglia remains unclear. Main purpose of the ventral nerve cord thus could be the innervation of the abdomen. As discussed above, females are sensitive to tactile stimuli at the abdomen and egg sacs, which are responded to by the retraction of the abdomen (Schrödl 1997, personal observation).

In contrast to the female, at least the first thoracopod of the male *I. aliena* shows strong musculature, but no distinct ventral nerve cord could be detected. Unfortunately in this area a few sections were lost in the male and therefore it is only concluded preliminarily that no well-developed ventral nerve cord exists in males. Since Belcik did not observe the nervous system (Belcik 1965, 1981), there is no comparable data of other splanchnotrophids available yet.

Purpose of the dorsal appendages

One of the most characteristic features of female splanchnotrophids are their dorsal appendages and several possible functions were suggested: O'Donoghue (1924) assumed an absorption of nutrients through the body wall with the appendages increasing the body surface. This was discussed by Huys (2001) and Anton & Schrödl (2013) to be rather unlikely because of the presence of functional mouthparts. As discussed above, the thin body cuticle may allow the absorption of dissolved nutrients, but we do not consider it as a major function of the dorsal appendages. Later Huys (2001) assumed the appendages to house parts of the ovary, which are visible looking at the translucent parasites. Herein we confirm the ovary reaching into the appendages by histological data (see Fig. 2). Optimising the space available for egg production is clearly a major function of splanchnotrophid appendages, especially when considering that rather soft and

slender appendages can fill available space in the body cavity of hosts without necessarily destroying certain organs or competing for space too fiercely (Anton & Schrödl 2013).

Huys (2001) also suggested the dorsal appendages to enwrap the viscera on which the female feeds. Anton & Schrödl (2013) discussed this function to be rather unlikely, since the dorsal appendages as newly built structures (Hancock & Norman 1863) would take over the function of the thoracopods, which are in turn reduced. We show herein that there are no muscles detectable inside the appendages and therefore no active movement is possible, making them rather inappropriate to maintain a feeding position. Evidence against tissue feeding is summarised above. Nevertheless, splanchnotrophid appendages can grow extremely long in some species and more or less irregularly enwrap viscera of hosts. This might result in acting as counterpart to body contraction, e.g. when an anchored female *I. aliena* retracts its abdomen with egg sacs creating a fold within the usually tough integument of the host (personal observation).

More recently, Salmen et al. (2008b) suggested the dorsal appendages to increase the respiratory surface. As it is characteristic for cyclopoid copepods (Kaestner 1967, Schram 1986, Schminke 2007) respiration is generally achieved by the gradient of oxygen concentration throughout the body integument (Wolvekamp & Waterman 1960, Ikeda et al. 2007). The present study confirms the absence of any respiratory organs in both sexes of *I. aliena*, which raises the question, why this increase of respiratory surface is only necessary in females. One reason could refer to ramified gonads themselves having large surfaces, possibly for ensuring supply with nutrients and oxygen. Another factor is size, of gonads and of the entire body: Kaestner (1967) stated that a parasitic lifestyle often leads to an excessive growth in female copepods due to an oversupply of food. Such a growth would – according to Kaestner – be restricted to the body and would leave the cephalic limbs remaining as small appendages to the allometrically enlarged body (Kaestner 1967). Size increase in females is exactly the case in all splanchnotrophid genera with thoracopods and dorsal appendages being also enlarged. Providing additional surface for respiration and volume for ovaries while minimizing harm to hosts would explain why these appendages are found in all female splanchnotrophids (Anton & Schrödl 2013) but never in males. In case of the Splanchnotrophidae thus the term “dwarf male” may be misleading as already assumed by Laubier (1966) and it may be more correct to speak of giant females.

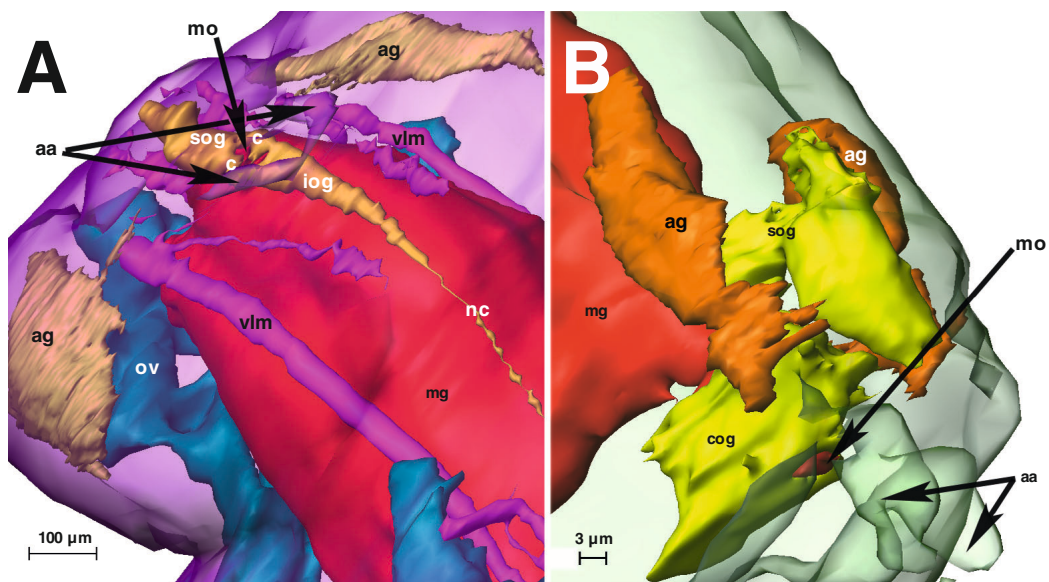


Fig. 6. Nervous system of both sexes of *I. aliena*. **A**. Female, ventral view; **B**. male latero-ventral view. aa, antennae; ag, antennal gland; c, connective; cog, circum-oesophageal ganglion; iog, infra-oesophageal ganglion; mg, midgut; mo, mouth; nc, nerve cord; sog, supra-oesophageal ganglion.

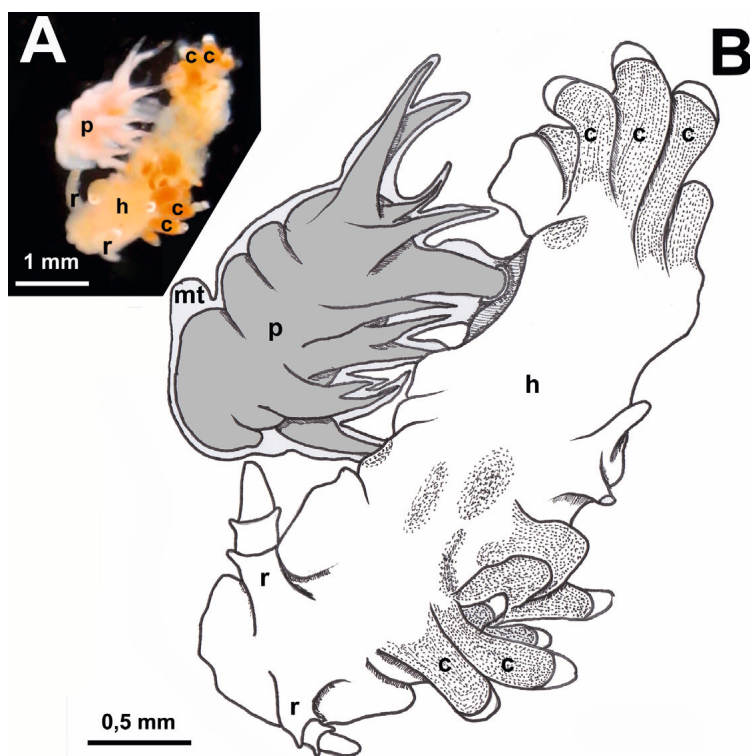


Fig. 7. Specimen of *Flabellina* sp. 1 (sensu Schrödl 2003) infected with one female *Ismailia*. The egg sacs of the parasite were removed for molecular analysis. **A**. Picture of the living animals. **B**. Drawing to clarify the position of the parasite inside the host (since the parasite is encapsulated by the integument of the host it has to be considered inside the host). Dorsal view of the host with a lateral view of the parasite flipped to the right. The abdomen of the female parasite is protruding the integument of the host and emerges on the ventral surface of the mantle. c, cerata; h, host; mt, mantle tissue surrounding the parasite; p, parasite; r, rhinophores.

Conclusion

The present study provides new insights concerning morphology, organ functions and life history of the genus *Ismaila*. To gain a comprehensive overview of the family Splanchnotrophidae it will nevertheless be inevitable to analyse the internal anatomy of representatives of the remaining genera, in addition to studying the genus *Briarella* Bergh, 1876. Especially *B. doliaris* Salmen, Anton, Wilson & Schrödl, 2010 shares many external features with the genus *Splanchnotrophus* (see Salmen et al. 2010) and may be of importance to this task. Detailed but time-consuming histology-based microanatomical 3D modelling as applied herein should be complemented by μ CT scanning; once histological structures are reliably correlated to μ CT scans the latter technique may prove efficient. In particular, tomography should provide comprehensive data on number, position and external morphology of cuticularised parasites and their larva within the hosts. Together with direct observations and experiments on e.g. potential chemotaxis and infection of hosts, which have proven difficult, conclusions from structural and functional evidence may be the key to understanding splanchnotrophid life cycles and behaviour. Molecular studies will allow for testing the current morphology-based species delimitations, and help to unravel the evolutionary history of such highly adapted parasites as the Splanchnotrophidae.

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