New host for the parasitic worm *Anoplodium* sp. (Rhabditophora: Platyhelminthes) found in the sea cucumber *Isostichopus fuscus* (Holothuroidea: Echinodermata)

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Abstract

A flatworm was discovered inside the coelomic cavity of the commercial sea cucumber *Isostichopus fuscus* along the Pacific coast of Mexico. Based on morphological and genetic evidence, it was determined to be *Anoplodium* sp. belonging to class Rhabditophora. Thus, the sea cucumber *I. fuscus* constitutes a new host. The flatworms were consistently found on the surface of the haemal vessels and the *rete mirabile* of 92% of the sea cucumbers sampled along the cost of Mazatlan, and 88% of the sea cucumbers collected in the Sea of Cortez. The infestation rate varied from 1 to 725 flatworms per individual, in both male and female sea cucumbers. When more than ~120 *Anoplodium* sp. were counted in a single host, the gonads of the latter were either very small (≤1.2 g wet weight, or GI <0.26) or absent, suggesting that the flatworm could be detrimental to *I. fuscus* and be considered parasitic. Combined with the threat of overfishing throughout its distribution range, the discovery of this parasite could seal the fate of *I. fuscus* in certain regions of the eastern Pacific.

Introduction

Sea cucumbers are known to host a variety of associated species that may dwell externally — on their body wall, around their mouth, and among their tentacles — or internally, inside the respiratory tree, intestines, or coelomic cavity (Jangoux 1987; Eeckhaut et al. 2004). In particular, sea cucumbers living in the Indo-Pacific and the Caribbean regions can host several species at a time and sometimes in considerable numbers. Crustaceans, gastropods, bivalves, and fishes are described either as epibionts or endobionts, and are considered to be either obligate or facultative associates of sea cucumbers. These associations are mostly determined to be commensal (e.g. Mercier and Hamel 2005) or parasitic (e.g. Heading and Mandahl-Barth 1938; Hamel et al. 1999). Over a decade ago, Eeckhaut et al. (2004) indicated that ~150 species of metazoans could be found in sea cucumbers, and this number is increasing every year.

Worms are commonly reported in association with sea cucumbers (Jangoux 1987; Eeckhaut et al. 2004), including members of classes Rhabditophora (e.g. *Anoplodium*, *Avagina*, *Cleistogomia*, *Syndesmis*), Trematoda (e.g. *Fellodistomun*, *Monorchis*, *Zoogonus*), Nematoda (*Ananus*, *Onchaleimus*, *Thalassonema*) and Polychaeta (e.g. *Ophryotrocha*, *Gastrolepia*). Among the associates of echinoderms, Jangoux (1987, 1990) described 58 Rhabdocoela.

Within the phylum Platyhelminthes, members of the order Rhabdocoela, and especially the genus *Anoplodium*, have been reported from various species of sea cucumbers spreading from polar to tropical habitats (e.g. Marcus 1949; Westblad 1953; Hyman 1960; Doignon et al. 2001). The number of associates found in each host varied between 1 and 202. For example, Doignon et al. (2001) found between 128 and 202 *Anoplodium parasitica* in the sea cucumber *Holothuria tubulosa*. The same species was also found to parasitise *H. polii* and *H. stellati*. These worms typically infest the coelomic cavity or the digestive tract of their hosts (Jangoux 1987). The species *Anoplodium hymanae* described by Shinn (1983, 1985a, b) can be found in the coelomic cavity of 88–94% of *Stichopus californicus* on the west coast of North America (Washington State, USA), with an average prevalence of 15 worms per sea cucumber. Shinn (1983) considers *Anoplodium hymanae* to be parasitic because it was found to consume a large number of host coelomocytes. Shinn (1985a, b) also described the reproduction and infestation mechanisms of the species.

The present work outlines a new host for a species of the genus *Anoplodium*, reports on its infestation

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rates, and discusses negative impacts on the host, supporting its classification as parasitic.

Materials and methods

Collection

Specimens of the sea cucumber *Isostichopus fuscus* were collected by divers along the coast of Mexico in the Sea of Cortez (25.59N: 110.88W; n=430 individuals) and off the coast of Mazatlan in western Mexico (23.27N: 106.63W; n=734 individuals) in June and July 2009 between 5 and 15 m depth.

Out of those, 45 sea cucumbers were analysed from the Sea of Cortez and 112 from Mazatlan. The contracted length (mouth–anus), whole wet weight and eviscerated body wall wet weight of each sea cucumber was measured. Sex was determined from a biopsy of the gonad and gamete smears examined under a light microscope. The gonad index was established as the percent wet weight of the gonads on the eviscerated body wall wet weight. The haemal system and the respiratory tree were carefully transferred to a Petri dish, and the coelomic cavity and the surface of the intestines were rinsed with filtered seawater. The flatworms present were counted and measured. Because individuals are slightly oval in shape (Fig. 1), two measurements were taken, one of the longest axis and the other of the axis perpendicular to it. Subsamples of the flatworms were preserved either in 100% ethanol (n = 300–350) for genetic analyses, or 4% formaldehyde (n~200) for histology and morphological description.

Histology and DNA extraction and sequencing

To identify and characterise the worm species, two different approaches were used. First, histological slides were prepared from preserved samples. Five formaldehyde-fixed samples and 5 ethanol-preserved samples were immersed in a 3% solution of glutaraldehyde in cacodylate buffer (0.1 M, pH 7.8) for 3 h at 4°C. They were then rinsed in the buffer and post-fixed for 1 h in a 1% solution of osmium tetroxide in the same buffer. After a final rinsing in the buffer, they were dehydrated in a graded ethanol series and embedded in Spurr resin. Serial transverse and longitudinal 1 µm thick sections were made with a glass knife using a Reichert Om U2 ultramicrotome, and stained in a 1:1 solution of methylene blue and azur II according to the method used by Humphrey and Pittman (1974).

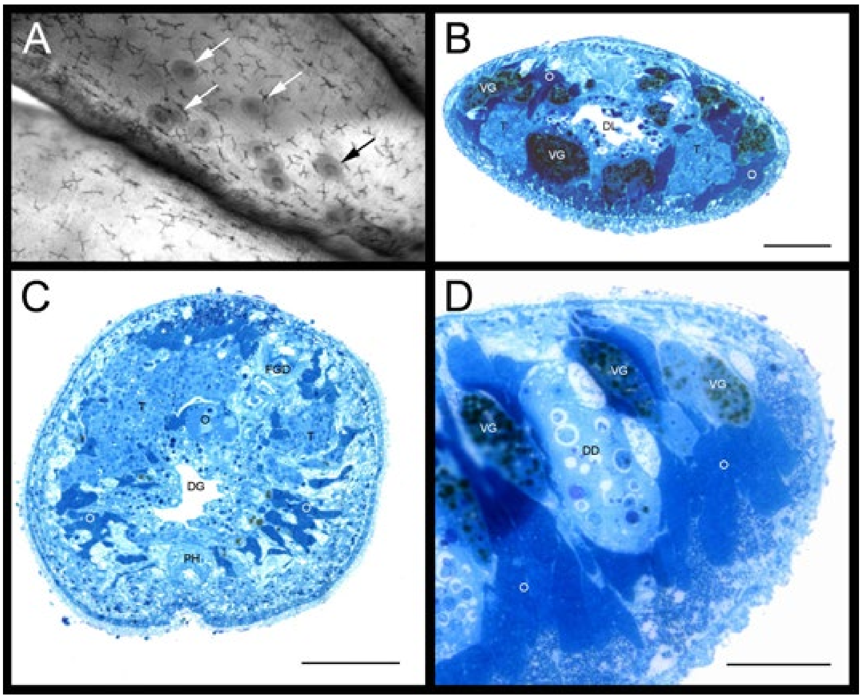

Figure 1. A. Live individuals (500–700 µm) of the worm *Anoplodium* sp. (arrows) on the *rete mirabile* of the sea cucumber *Isostichopus fuscus*. B. Transverse section in the middle part of the body. C. Frontal section in the ventral part of an individual. D. Details of the internal tissues (longitudinal section). Scale bar in B and C represents 100 µm, scale bar in D represents 50 µm. DD = digestive diverticula; DL = digestive lumen; FGD = female genital ducts; O = ovary; PH = pharynx; T = testicle; VG = vitelline gland.

Secondly, identification was made using 18S rDNA sequencing. Five ethanol-preserved samples were pooled together, and their genomic DNA extracted with the commercial kit Invitek Spin Tissue Mini kit (Invisorb). DNA fragments from the 18S rDNA (ca. 1,200 nucleotides in all) were amplified by a polymerase chain reaction and sequenced following the detailed procedure described in Eeckhaut et al. (2000) and Lanterbecq et al. (2006). The sequences obtained were processed and cleaned using the program code Codon Align, and subjected to a BLAST (Basic Local Alignment Search Tool) search in the National Center for Biotechnology Information database. Two portions of the 18S rDNA of ca. 600 bp each were deposited in Genbank under accession numbers KY320456 and KY320457.

Results and discussion

Species identification

Based on morphological characteristics, the flatworm that was found to infest the commercial sea cucumber *Isostichopus fuscus* in both locations along the coast of Mexico was identified as *Anoplodium* sp. (Platyhelminthes: Rhabdocoela). Two 18S rDNA fragments of 640 and 555 p long were obtained and checked against the BLAST database, which confirmed that the species belonged to the genus *Anoplodium*, with 95% and 93% similarity to *Anoplodium stichopi* (Table 1).

Table 1. The seven closest related species according to BLAST searches on the two 18S rDNA fragments of the parasite, with the percentage of identity of the fragments, and the accession numbers of the related sequences.

Most related species	Fragment 1	Fragment 2	Accession number
Anoplodium stichopi	96	94	AF167424.1
Seritia elegens	93	92	KC529517.1
Wahlia macrostilifera	92	91	KC529518.1
Veidovskya ignava	90	87	KC529513.1
Veidovskya pellucida	90	87	KC529512.1
Provortex balticus	92	86	KC529511.1
Provortex karingi	92	85	KC529510.1

However, the latter species is from the Mediterranean Sea. Sequences from *A. hymanae (US West Coast)* are not available for optimum comparison with Shinn (1983). However, the morphology of the specimens found here differs markedly from previous descriptions, suggesting a new species of *Anoplodium*. Specimens were deposited at the Department of Ocean Sciences, Memorial University (Canada).

The flatworm's body is flat and longer than it is wide, with the anterior end slightly truncated. The samples were characterised by two main size classes. The largest specimens were between 750 ± 75 μm long and 690 ± 55 μm wide (comprising ~29% of the flatworms collected) and the smallest individuals were 590 ± 25 μm long and 500 ± 25 μm wide (Fig. 1) comprising the majority of individuals. Compared with the maximum size of *A. hymanae* described by Shinn (1983), which were 2.0–2.5 mm long, specimens in the present study are clearly smaller. Live specimens were white or greyish and the body wall was transparent (Fig. 1a). Histological sections showed a uniform ciliated epidermis, a blind digestive system with caeca and a pharynx opening ventrally, and two testicles extending along both sides of the body. The main elements of the female genital systems observed on sections were the vitelline glands and what we suppose are two ovaries that also extend along both sides of the body.

Prevalence and infestation rates

Between 88% and 92% of all sea cucumbers from Mexico hosted the Rhabdocoela *Anoplodium* sp. in the present study, exhibiting prevalence rates similar to those reported by Shinn (1983) for *A. hymanae* in *Stichopus californicus* (89–94%). However, the prevalence rate is high relative to other infestation rates recorded for the same genus; for example, 13% of *Holothuria tubulosa* were reportedly infested by *A. parasitica* in the Mediterranean Sea (Doignon et al. 2001). Furthermore, a maximum of ~750 flatworms per host were found in the present study, much more than the 15 flatworms described in *S. califiornicus* by Shinn (1983). Infestation rates of ~200 flatworms per host were found in some tropical sea cucumbers from Papua New Guinea (Eeckhaut et al. 2004). Overall, the present study represents the highest number of *Anoplodium* per host ever observed in a sea cucumber (Jangoux 1987). The extreme case of flatworm infestation recorded here could be attributed to the fact that *I. fuscus* at both locations occurred in highly populated and polluted areas along the coast (e.g. sewage discharge). The water quality could have stimulated the proliferation of coelomocytes and indirectly lead to favourable food sources for the parasites, thereby enhancing their multiplication.

Shinn (1983, 1985a, b) reported that that *A. hymanae* competed with its host for nutrients and thus may elicit adverse side effects. He demonstrated that the flatworms could consume coelomocytes in *Stichopus californicus*, and concluded that *A. hymanae* was indeed parasitic. The present study showed a drop in the host's gonad size (≤1.2 g wet weight) when the number of *Anoplodium* sp. reached ≥120 individuals per host. This castrating effect was recorded in both male and female sea cucumbers. Non-infested sea cucumbers displayed gonad index values between 6.3 and 12.4 compared with a maximum of 0.24 in infested individuals. An overabundance of flatworms in the coelomic cavity may deplete the coelomocytes to the point of preventing gonad growth, supporting the classification of *Anoplodium* sp. as a parasite of the sea cucumber *I. fuscus*.

Effect on host and conservation concerns

In the present study, *Anoplodium* sp. was found to occur on the surface of the haemal vessels and the *rete mirabile*. Individuals occurred mainly in small groups of 3 to 12 and were sometimes overlapping each other (Fig. 1a). When removed from the coelomic cavity of the sea cucumber host, *Anoplodium* sp. did not survive more than ~2 h in ambient seawater.

Anoplodium sp. colonised both male and female sea cucumbers.

The sea cucumber *Isostichopus fuscus* is the second most expensive sea cucumber on the market, just after *Apostichopus japonicus*; it is fished throughout its distribution range and has been listed as endangered on the International Union for Conservation of Nature Red List (Mercier et al. 2013). Therefore, the presence of the parasite *Anoplodium* sp. could exacerbate the threat of overfishing in certain areas of the eastern Pacific, through a significant decrease in reproductive output. This finding suggests that the presence of *Anoplodium* sp. in fished populations of *I. fuscus* should be monitored closely as part of the management plan; otherwise, the species could suffer local extinctions in the near future, at least in heavily infested areas along the coast of Mexico. Future investigations should seek to determine whether this parasite occurs in other areas of the distribution range of *I. fuscus*, which extends as far south as Ecuador.

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References

Doignon G., Jangoux M., Féral J.P. and Eeckhaut I. 2001. Seasonal release of the egg capsules of *Anoplodium parasita* Schneider, 1858, intracoelomic turbellarian (Platyhelminthes, Rhabdocoela) symbiotic of the sea cucumber *Holothuria tubulosa* Gmelin, 1788 (Echinodermata, Holothuroida). Echinoderm Research 200:261–264.

Eeckhaut I., McHugh D., Mardulyn P., Tiedemann R., Monteyne D., Jangoux M. and Milinkovitch M.C. 2000. Myzostomida: A link between trochozoans and flatworms? Proceedings of the Royal Society of London B 267:1–10.

Eeckhaut I., Parmentier E., Becker P., Gomez da Silva S. and Jangoux M. 2004. Parasites and biotic diseases in field and cultivated sea cucumbers. FAO Fisheries Technical Paper 463:311–325.

Heading S.G. and Mandahl-Barth G. 1938. Investigations on the anatomy and systematic position of the parasitic snail *Entocolax voigt*: 6. og 7. Thule-Expedition til Sydøstgrønland 1931–1933: Reitzel.

Hamel J.-F., Ng P.K. and Mercier A. 1999. Life cycle of the pea crab *Pinnotheres halingi* sp. nov., an obligate symbiont of the sea cucumber *Holothuria scabra* Jaeger. Ophelia 50(3):149–175.

Humphrey C.D. and Pittman F.E. 1974. A simple methylene blue-azure II-basic fuchsin stain for epoxy-embedded tissue sections. Stain Technology 49(1):9–14.

Hyman L. 1960. New and known umagillid rhabdocoels from echinoderms. American Museum Novitaes 1984:1–14.

Jangoux M. 1987. Diseases of Echinodermata. II. Agents metazoans (Mesozoa to Bryozoa). Diseases of Aquatic Organisms 2:205–234.

Jangoux M. 1990. Diseases of Echinodermata. p. 439–567. In: Diseases of Marine Animals. Kinne O. (ed). Vol. 3. Biologischen Ansalt Helgoland, Hamburg.

Lanterbecq D., Rouse G.W., Milinkovitch M.C. and Eeckhaut I. 2006. Molecular phylogenetic analyses indicate multiple independent emergences of parasitism in Myzostomida (Protostomia). Systematic Biology 55(2):208–227.

Marcus E. 1949. *Turbellaria brasileiros* (7). Boletins da Faculdade de Philosophia, Sciencias e Letras, Universidade de São Paulo. Zoologia 14:7–156.

Mercier A. and Hamel J.-F. 2005. Note on the association between *Plakobranchus ocellatus* (Mollusca: Gastropoda: Opisthobranchia) and *Holothuria atra* (Echinodermata: Holothuroidea). Cahiers de Biologie Marine 46:399–402.

Mercier A., Hamel J.-F., Toral-Granda T.-G., Alvarado J.J., Paola Ortiz E. and Benavides M. 2013. *Isostichopus fuscus*. The IUCN Red List of Threatened Species 2013:e.T180373A1621878. Available at: http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T180373A1621878.en (Accessed 20 July 2016).

Shinn G.L. 1983. *Anoplodium hymanae* sp. n., an umagillid turbellarian from the coelom of *Stichopus californicus*, a Northeast Pacific holothurian. Canadian Journal of Zoology 61(4):750–760.

Shinn G.L. 1985a. Reproduction of *Anoplodium hymanae*, a turbellarian flatworm (Neorhabdocoela, Umagillidae) inhabiting the coelom of sea cucumbers; production of egg capsules, and escape of infective stages without evisceration of the host. The Biological Bulletin 169(1):182–198.

Shinn G.L. 1985b. Infection of new hosts by *Anoplodium hymanae*, a turbellarian flatworm (Neorhabdocoela, Umagillidae) inhabiting the coelom of the sea cucumber *Stichopus californicus*. The Biological Bulletin 169(1):199–214.

Westblad E. 1953. New turbellaria parasites in echinoderms. Arkiv för Zoologi 5:269–288.