PARINT-2016-37

High-intensity cardiac infections of *Phthinomita heinigerae* n. sp. (Digenea: Aporocotylidae) in the orangelined cardinalfish, *Taeniamia fucata* (Cantor), off Heron Island on the Great Barrier Reef

Matthew J. Nolan [a, *], Cinzia Cantacessi [b], Scott C. Cutmore [c], Thomas H. Cribb [c], and Terrence L. Miller [d, e,]

[a] *Department of Pathology and Pathogen Biology, Royal Veterinary College, University of London, North Mymms, Hatfield AL9 7TA, United Kingdom*

[b] *Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, United Kingdom*

[c] *School of Biological Sciences, The University of Queensland, St Lucia 4072, Australia.*

[d] *Centre for Sustainable Tropical Fisheries and Aquaculture, School of Marine and Tropical Biology, James Cook University, PO Box 6811, Cairns 4878, Australia*

[e] *Fish Health Laboratory, Department of Fisheries Western Australia, 3 Baron-Hay Court, South Perth 6151, Australia*

* *Corresponding author at:* the Department of Pathology and Pathogen Biology, Royal Veterinary College, University of London, Hatfield, Hertfordshire AL9 7TA, United Kingdom. Tel.: +44 (0) 1707 66 6803. Email: mnolan@rvc.ac.uk (M.J. Nolan).

Email addresses:

MJN: mnolan@rvc.ac.uk

CC: cc779@cam.ac.uk

SCC: scott.cutmore@uqconnect.edu.au

THC: t.cribb@uq.edu.au

TLM: terry.miller@fish.wa.gov.au

ABSTRACT

We report a new species of aporocotylid trematode (Platyhelminthes: Digenea) from the heart of the orangelined cardinalfish, *Taeniamia fucata* (Cantor), from off Heron Island on the southern Great Barrier Reef. We used an integrated approach, analysing host distribution, morphology, and genetic data from the internal transcribed spacer 2 of the ribosomal DNA, to circumscribe *Phthinomita heinigerae* n. sp. This is the first species of *Phthinomita* Nolan & Cribb, 2006 reported from the Apogonidae; existing species and known 'types' are recorded from species of the Labridae, Mullidae, and Siganidae. The new species is distinguished from its 11 congeners in having a body 2977–3539 long and 16.5–22.4 times longer than wide, an anterior testis 6.2–8.2 times longer than wide and 8.3–13.0 times longer than the posterior testis, a posterior testis whose width is 35–56% of the body width, and an ovary positioned 11–13% of the body length from the posterior end. *P. heinigerae* n. sp. differs further in having an ovary that is positioned entirely anterior to the posterior margin of the anterior testis. In addition, 2–34 base differences (0.4–7.0% sequence divergence) were detected among the ITS2 sequence representing *P. heinigerae* n. sp. and the 14 representing other *Phthinomita* species/molecular types. Prevalence and intensity of infection with *P. heinigerae* n. sp. was relatively high within the heart tissue of *T. fucata*, with 19 of 20 fish examined from off Heron Island infected (95%) with 7–25 adult worms (arithmetic mean 16.6). Infections by these parasites accounted for an occupation of 7–30% of the total estimated heart volume.

Keywords

Platyhelminthes

Trematoda

Apogonidae

Internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA)

Host-switching

1. Introduction

The Aporocotylidae Odhner, 1912 (Platyhelminthes: Trematoda) is a family of parasitic flatworms that has, in recent years, emerged as an increasingly rich, and morphologically diverse, group of digeneans. There are currently 142 accepted species from 37 genera [1-5], which infect a broad range of fishes. Species from seven genera have been recorded from fishes of the Great Barrier Reef (GBR): *Ankistromeces* Nolan & Cribb, 2004 (see [6]); *Braya* Nolan & Cribb, 2006 (see [7]); *Cardicola* Short, 1953 (see [7-9]); *Pearsonellum* Overstreet & Køie, 1989 (see [10, 11]); *Plethorchis* Martin, 1975 (see [12]); *Phthinomita* Nolan & Cribb, 2006 (see [6]); and, *Rhaphidotrema* Yong & Cribb, 2011 (see [13]). *Phthinomita* is the most complex of these, consisting of 11 recognised species and numerous undefined 'types' represented by a unique DNA sequence or single morphological specimen. Unlike most aporocotylids, which are typically characterised by a flat body that may be linear, elliptical, or lanceolate, species of *Phthinomita* are long and thread-like. As adults, they wind through the intertrabecular spaces of the ventricle of their hosts, which to date include species of labrid (wrasses), mullid (goatfishes), and siganid (rabbitfishes or spinefoots). Due to the extreme morphological similarity that exists among species of *Phthinomita*, an effect most likely due to their site of infection, this group is best described as a complex of cryptic species. As such, genetic data are required to enhance traditional methods of species characterisation (i.e. microscopic and morphological examination, host and geographic distribution) and the delineation of species is only possible though this integrated approach (see [14]). Here, we report *Phthinomita heinigerae* n. sp. from the ventricle of the orangelined cardinalfish, *Taeniamia fucata* (Cantor) (Perciformes: Apogonidae), collected during the CReefs project from 2009–2012 (http://www.aims.gov.au/creefs/field-program.html), from off Heron Island on the southern GBR.

2. Materials and methods

2.1. Sample collection

Between 2009 and 2012, 22 species of apogonid from nine genera (Table 1) were collected from five sites off Heron Island on the southern GBR (23.4420° S, 151.9140° E), eight sites off Lizard Island on the northern GBR (14.6680° S, 145.4617° E), and from seven sites on Ningaloo reef, off Western Australia (22.5625, 113.810278). Apogonid fishes were stored in an 80 litre container before being euthanised by an overdose of clove oil, in strict accordance with the Queensland Museum's Animal Ethics Permit 07/01, issued for this research. Immediately upon death the heart, gills, and viscera were excised and processed as described previously [8]. The hearts of infected apogonids were preserved in 10% formalin (room temperature), for histological examination.

2.2. Morphological examination of aporocotylids

Fixed worms were washed, stained, and mounted as described by Nolan et al. [8]. Drawings were completed using a drawing tube attached to an Olympus BX53 compound microscope with Nomarski differential interference contrast (DIC) optics. We inferred the dorsal surface by reference to the position of the separate genital pores, which were assumed to be dorsal, as in all *Phthinomita* species. All measurements, in micrometres, were made using an Olympus UC50 digital camera and the software LabSens (Olympus Soft Imaging Solutions), and are presented as a range followed by the arithmetic mean in parentheses. Measurement of morphological characters from the anterior or posterior end of worms reflects the distance from the extremities of each feature. Caecal lengths as a percentage of body length are based on the right caeca only. Type-specimens, hologenophores, and paragenophores were deposited in the Queensland Museum, Australia (QM).

2.3. Isolation of genomic DNA, Polymerase chain reaction, and phylogenetic analysis

Total genomic DNA (gDNA) was isolated from three separate specimens identified morphologically as putative *P. heinigerae* n. sp. using a DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR amplification of the entire internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA) region was achieved using the primers 3S (forward: 5'-GGTACCGGTGGATCACGTGGCTAGTG-3') and ITS2.2 (reverse: 5'-CCTGGTTAGTTTCTTTTCCTCCGC-3'). PCR was carried out in a 20 µl volume as described by Cutmore et al. [15]. All resultant PCR amplicons were purified and sequenced as described by Nolan et al. [8].

Prior to phylogenetic analysis, the sequence representing *P. heinigerae* n. sp. (GenBank accession no. KX168409) was aligned with 30 reference sequences for selected aporocotylid species/genera, presently available in GenBank. Sequences were aligned using the software MUSCLE version 3.7 [16, 17] with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. The resultant alignment was adjusted manually using the software BioEdit [18]. Total nucleotide distance matrices were calculated using the pairwise deletion of gaps/missing data option in the software package MEGA v.5 [19].

Formatted: Highlight

Minimum evolution analysis was conducted on the ITS2 dataset using MEGA v.5. Nodal support for the analysis of this dataset was inferred by bootstrap analysis using a heuristic search of 10,000 replicates. The outgroup taxa used were six species/molecular types of *Ankistromeces* (GenBank accession nos. DQ335838–DQ335843, [6]).

2.4. Histology

Sections (5 µm thick) were cut from 10% formalin fixed tissue samples as described by Heiniger et al. [20]. In brief, tissue sections were stained with haematoxylin and eosin (H & E). Coverslips were applied using DePeX (BDH, England). Digital, light microphotographs of the sections were taken at ×100 magnification using an Olympus UC50 digital camera attached to an Olympus BX53 compound microscope.

2.5. Estimating volume of heart space occupied by P. heinigerae *n. sp. in infected* T. fucata

To estimate the volume of individual worms, additional measurements were taken from each type specimen (n = 9). Because blood flukes vary in diameter over the length of the body, between six and 11 radius and height (i.e. length) measurements were taken and used in the formula for calculating the volume of a cylinder ($V = \pi r^2 h$, where r is the radius and h is the height). The general body morphology of species of *Phthinomita* are more cylindrical than dorso-ventrally flattened, therefore these volume calculations were considered appropriate. These were then combined to obtain the approximate total volume for each type specimen, and then averaged to obtain the arithmetic mean volume of a single worm. To approximate the volume of a *T. fucata* heart, whole formalin fixed hearts (n = 3) were measured and the radius of each used in the formula to calculate the volume of a sphere ($V = 4/3\ \pi r^3$). Using the average volume of a worm, the percentage volume of heart 'space' occupied, based on the minimum (n = 7), mean (16.6), and maximum (25) intensities observed, was estimated.

3. Results

3.1. Aporocotylid prevalence and specificity

The hearts of 19 of the 724 apogonid specimens examined (2.6%) were infected with thread-like aporocotylids. All 19 infected individuals were identified as the orangelined cardinalfish, *T. fucata*, which were all collected from a single site in the Heron Island lagoon (19/20; 95% prevalence); none of the 27 *T. fucata* specimens collected from two sites off Lizard Island (Casuarina beach and Turtle beach) were infected.

3.2. Morphology

Class Trematoda Rudolphi, 1808
Subclass Digenea Carus, 1863
Order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003
Suborder Diplostomata Olson, Cribb, Tkach, Bray & Littlewood, 2003

Superfamily Schistosomatoidea Stiles & Hassall, 1898

Family Aporocotylidae Odhner, 1912

Phthinomita Nolan & Cribb, 2006

3.3. P. heinigerae n. sp.

Description and measurements (Figs. 1–2): (based on nine whole mature worms). With all features of genus. Body slightly notched at male genital pore, 2977–3539 (3249) × 133–198 (167), 16.5–22.4 times longer than wide. Oral sucker weakly developed, bearing concentric rows of fine spines. Oesophagus straight, 698–887 (783) or 22–25% of body total length. Intestine; right anterior caecum 25–56 (37) or 0.8–1.7% of body total length, left anterior caecum 25–54 (35) long; posterior caeca sinuous, unequal, irregular in outline; right posterior caecum 601–948 (758) or 18.9–31.8% of body total length; left posterior caecum 882–1075 (977) long; 12.4–37.9 times longer than anterior pair.

Anterior testis originating posterior to intercaecal field, but antero-dextral to distal termination of left posterior caecum (see Fig. 1), containing dorso-ventral muscle fibres, 844–1206 (1068) or 28–37% of body total length × 130–185 (155) or 83–99% of body total width, 6.2–8.2 times longer than wide, 8.3–13.0 times longer than posterior testis; posterior testis ovoid, rudimentary, 76–114 (99) or 3–4% of body total length × 55–93 (76) or 35–56% of body total width, 1.0–1.6 times longer than wide. Vas deferens seen antero-dextral to posterior margin of anterior testis; duct from posterior testis passing antero-medially. Cirrus-sac tear-shaped, 34–54 (44) × 24–42 (31); 1.3–1.9 times longer than wide; 191–243 (219) from posterior extremity, 6–7% of body total length. Internal seminal vesicle ovoid, occupying posterior end of cirrus-sac; ejaculatory duct sinuous; prostatic cells not seen.

Ovary spherical to ovoid, entirely anterior to posterior margin of anterior testis (see Fig. 2), 351–435 (394) or 11–13% of body total length from posterior extremity, 68–99 (84) or 2–3% of body total length × 72–94 (82) or 40–60% of body total width. Oviduct originating at posterior dorsal margin of ovary, dorsal to vas deferens, entering oötype postero-dorsally. Vitelline duct forming lateral to ovary, posteriorly dextral to vas deferens and cirrus-sac. Oötype ovoid, 35–47 (42) × 18–24 (21). Mehlis' gland extending anteriorly to posterior margin of cirrus-sac, and posteriorly to anterior margin of posterior testis. Uterus extending from oötype sinuously, sinistral to oviduct. Uterine chamber forming posterior to posterior margin of ovary, sinuous, curving dorsally posteriorly to female pore, 144–185 (171) × 23–39 (33). Eggs 14–22 (18) × 8–15 (10) (n = 10). Vitelline follicles extending anteriorly past intestinal bifurcation, sinistral and dextral to oesophagus, posterior caeca and anterior testis, posteriorly extending to anterior margin of ovary.

3.4. Taxonomic summary

Type-host: *Taeniamia fucata* (Cantor), the orangelined cardinalfish (Perciformes: Apogonidae).

Type-locality: Heron Island lagoon, Heron Island (23.4420° S, 151.9140° E), southern Great Barrier Reef, Queensland, Australia.

Site: Intertrabecular spaces and lumen of ventricle (heart).

Intensity: 7−25 (arithmetic mean 16.6).

Prevalence: 19 of 20 (95%).

Type-material: Holotype (QM G ~~XXXXXX~~232105), eight paratypes (QM G ~~XXXXXX~~232106-232113).

Molecular sequence data: ITS2 (complete), three identical replicates.

Molecular voucher data: Hologenophore (QM G 232114), two paragenophores (QM G 232115-232116).

GenBank accession number: KX168409~~XXXXXX~~.

Etymology: Specific name '*heinigerae*' is in reference to our esteemed colleague Dr Holly Heiniger, for whom the initial samples of this species of Apogonidae were collected.

Formatted: Font: Italic

Formatted: Indent: Left: 1.27 cm

Formatted: Not Highlight

3.5. Molecular data

Three replicate ITS2 sequences were generated from as many specimens of *P. heinigerae*, all of which were identical. Comparison of the sequence represented by KX168409 ~~XXXXXX~~ with reference data for aporocotylids available on GenBank indicated this sequence type to be new, and two nucleotides different (0.4% sequence divergence over 485 base positions) from the most similar available sequence, represented by DQ335856 [6], which corresponds to *Phthinomita munozae* Nolan & Cribb, 2006 from *Choerodon venustus* (De Vis) (Labriformes: Labridae).

Phylogenetic analysis of 31 sequences (including outgroups) aligned over 485 positions (trimmed to match the shortest sequence length) resulted in a phylogram where species of *Phthinomita* formed a monophyletic clade, to the exclusion of the outgroup taxa (i.e. members of the genus *Ankistromeces*). The sequence representing *P. heinigerae* n. sp. grouped with the sequence represented by DQ335856, for *P. munozae*, as expected based on sequence comparisons. These sequences resolved as a strongly supported monophyletic clade together with sequences representing *Phthinomita poulini* Nolan & Cribb, 2006 (DQ335857–DQ335859) from *Parupeneus barberinus* (Lacepède) (Perciformes: Mullidae), *Parupeneus bifasciatus* (Lacepède) [now *Parupeneus trifasciatus* (Lacepède)], and *Parupeneus cyclostomus* (Lacepède), and *Phthinomita* sp. B (DQ335863) from *Mulloidichthys vanicolensis* (Valenciennes) (Mullidae) (see Fig. 3). As a result, species of *Phthinomita* that parasitise siganids represent a paraphyletic group. With the exception of sequences representing *Phthinomita littlewoodi* Nolan & Cribb, 2006, *Phthinomita hallae* Nolan & Cribb, 2006, and *Phthinomita jonesi* Nolan & Cribb, 2006 (bootstrap value = 69) components of the siganid-infecting species generally formed several well-supported clades (bootstrap values = 82–100). Although sequences did not group based on host or geographic distribution, basal *Phthinomita* species are mainly more 'robust' morphs relative to the smaller, more delicate *P. littlewoodi*, *P. hallae*, and *P. jonesi*.

3.6. Pathology

Specimens of *P. heinigerae* n. sp. were found in the lumen and intertrabecular spaces of the ventricle of *T. fucata* during autopsy and in histological sections of heart tissue. Fig. 4 shows a partially dissected heart from a single *T. fucata*. Infections included between 7–25 adult worms, which may each have a volume of between 1.85e+07–3.76e+07 (arithmetic mean 2.74e+07) μm^3 (see Section 2.5.). Based on the minimum and maximum number of worms found in a single infection, this could account for between 7–30% of the total estimated heart volume of *T. fucata*. Fig. 5 shows both transverse and (partial) longitudinal sections of *P. heinigerae* n. sp. within the heart. Cross-sections of *P. heinigerae* n. sp. were identified by the presence of cells within which different parts of the male (i.e. testes) and female (i.e. oötype) genitalia, and the caeca were recognised (see Fig. 5). No direct pathological changes produced by adult worms were detected in the heart tissue of infected fishes.

We did not observe eggs in the gills and/or the heart tissue of infected hosts.

4. Discussion

4.1. Taxonomy

Phthinomita heinigerae n. sp. can be differentiated from all current species of *Phthinomita* by the combined possession of a body 2977–3539 long and 16.5–22.4 times longer than wide, an anterior testis that is 6.2–8.2 times longer than wide and 8.3–13.0 times longer than the posterior testis, a posterior testis whose width is 35–56% of the body width, and having the ovary positioned 11–13% of the body length from the posterior end (see Table 2). In addition, *P. heinigerae* n. sp. differs further from all 11 species in having an ovary that is positioned entirely anterior to the posterior margin of the anterior testis (see Fig. 2); *P. robertsthomsoni* Nolan & Cribb, 2006 and *P. poulini* Nolan & Cribb, 2006 both possess an ovary that is positioned so that the posterior margin of the anterior testis passes adjacent to the ovary's medial line, while the remaining nine species all possess an ovary that is entirely posterior to, abutting, or only slightly overlapping the posterior margin of the anterior testis. *P. heinigerae* n. sp. is different from *Phthinomita brooksi* Nolan & Cribb, 2006 in having vitelline follicles that extend anteriorly past the intestinal bifurcation, and from *P. symplocos* Nolan & Cribb, 2006, *P. brooksi*, *P. hallae*, *P. jonesi*, *P. littlewoodi*, and *P. sasali* Nolan & Cribb, 2006 in having an anterior testis that overlaps the posterior margin of the posterior caeca (see Fig. 1).

Due to the general lack of morphological variation observed among species of *Phthinomita*, preceding work on this genus (and *Ankistromeces*) (see [6]) placed substantial weight on genetic data. To achieve this, a total of 135 sequences, with between one to 17 replicates for 30 host species/parasite species/geographical location combinations, was assembled to provide a robust dataset. Nineteen distinct

ITS2 genotypes were separated by 1–41 base differences (0.3–12.7% sequence divergence). These data showed that species of *Phthinomita* could be distinguished by as little as a single base difference (see page 69 in [6]). Here, sequence comparisons again confirmed that the specimens described as *P. heinigerae* n. sp. are distinct from the 11 recognised and three putative species of *Phthinomita* (2–34 base differences or 0.4–7.0% sequence divergence over 485 positions). These genetic differences, in combination with the morphological distinctions described above, and the host family, are consistent with *P. heinigerae* n. sp. being a new species.

4.2. Host specificity and prevalence of infection

This study is the first to report a species of *Phthinomita* from an apogonid fish. Existing species and known 'types' are largely restricted to the Mullidae [seven species - *Mulloidichthys vanicolensis*, *Parupeneus barberinoides* (Bleeker), *P. barberinus*, *P. bifasciatus*, *P. cyclostomus*, *P. indicus* (Shaw), and *P. multifasciatus* (Quoy & Gaimard)] and the Siganidae [nine species - *Siganus argenteus* (Quoy & Gaimard), *S. corallinus* (Valenciennes), *S. doliatus* Guérin-Méneville, *S. fuscescens* (Houttuyn), *S. lineatus* (Valenciennes), *S. puellus* (Schlegel), *S. punctatus* (Schneider & Forster), *S. virgatus* (Valenciennes), and *S. vulpinus* (Schlegel & Müller)] in the Indo-West Pacific Ocean [6]. One species, *P. munozae*, has been recorded from a labrid fish (*Choerodon venustus*). Although we dissected 724 specimens (n = 1–274) of 22 species of apogonid, *P. heinigerae* n. sp. was absent in all species but *T. fucata*. These specimens included 52 individuals of five species collected from the same patch reefs in the Heron Island lagoon where infected *T. fucata* were sampled. Similarly strict host specificity has been reported for *Kudoa leptacanthae* Heiniger & Adlard, 2012 (see [21]) (Multivalvulida: Kudoidae) from the apogonids *Zoramia leptacantha* (Bleeker) (74% or 199/269) and *Z. viridiventer* Greenfield, Langston & Randall (82.4% or 61/74) from off Lizard Island. Heiniger and Adlard [21] suggested this high prevalence of infection and the high host specificity might be explained by apogonid developmental biology (i.e. life cycle completion in a single lagoon, recruitment to home reefs, habitat specialists that are site attached and specific; see [22-28]). Furthermore, these authors proposed that the two-host life cycle of *K. leptacanthae* could be facilitated by the continual cycling of life stages through an intermediate host (presumably an annelid) in close proximity to home patch reefs. Given the two-host life cycle of marine teleost aporocotylids, which also incorporates an annelid intermediate host (e.g. [29-32]), similar reasoning for the high host and site specificity, and the high prevalence of infection, could be applied here.

In our phylogenetic analysis, *P. heinigerae* n. sp. and *P. munozae* (from a labrid) formed a well-supported clade together with the two mullid-infecting species of *Phthinomita*. As such, all the non-siganid infecting species form a clade exclusive to the siganid-infecting species, which form a paraphyletic assemblage. The most parsimonious explanation of this distribution is that the non-siganid clade arose as a host-switch from siganids. Host-switching is presumably difficult within this group as demonstrated by the general fidelity to the Siganidae. The topology of our analysis suggests that,

following the initial host-switch out of the Siganidae, the clade has adopted three distinct and only distantly related fish groups – Apogonidae, Labridae, and Mullidae. Although all three families have traditionally been considered members of the Perciformes, the Labridae are now considered by some to belong to a separate order, the Labriformes (see Figs. 9 and 10 in [33]). Comparable evidence of host-switching was reported by Nolan et al. [8], who showed that the lutjanid-infecting aporocotylids *Cardicola beveridgei* Nolan, Miller, Cutmore, Cantacessi, & Cribb, 2014 and *C. milleri* Nolan & Cribb, 2006 formed a well-supported clade with the chaetodontid-infecting *C. chaetodontis* Yamaguti, 1970. Similarly, Trieu et al. [34] showed that several apogonids (including *T. fucata*) shared a bivesiculid trematode, the sister species of which occurs in an unrelated pomacentrid. All three cases are evidence of the importance and recent history of host-switching by trematodes of coral reef fishes.

4.3. Pathology

Histological sections of heart tissue suggests that *P. heinigerae* n. sp. does not elicit immunological responses from *T. fucata* (see Fig. 5). Pathological changes induced by adult aporocotylids are rare, in contrast to the effects stimulated by accumulated eggs and escaping miracidia (see [35-43]). Overstreet and Thulin [44] found *Pearsonellum corventum* Overstreet & Køie, 1989 evoked an increased abundance of melanomacrophage centers in the heart of *Plectropomus leopardus* (Lacepède), while Herbert et al. [45] and Herbert and Shaharom [46] found *Cruoricola lates* Herbert, Shaharom-Harrison & Overstreet, 1994 and *Parasanguinicola vastispina* Herbert & Shaharom, 1995 (respectively) do not elicit pathological changes to infected blood vessels in *Lates calcarifer* (Bloch), despite *P. vastispina* possessing large spines that push into the endothelial cell walls (albeit they do not penetrate them). In contrast, Kirk et al. [38] found that attachment of adult *Sanguinicola inermis* Plehn, 1905 to vessel walls in the carp, *Cyprinus carpio* Linnaeus, caused hyperplasia of the endothelial lining and the occlusion of blood flow. More recently, Alama-Bermejo et al. [47] reported *Skoulekia meningialis* Alama-Bermejo, Montero, Raga, & Holzer, 2011 induced a *"localised, mild but chronic inflammatory response"* (see Fig. 5F, G, and H in [47]) in the ectomeninx of the meninges involving lymphocytes, macrophages, and eosinophilic granulocytes, together with clotted erythrocytes in the meningeal vessels of *Diplodus vulgaris* (Geoffroy Saint-Hilaire). Despite these exceptions, it is conceivable that adult aporocotylids, including *P. heinigerae* n. sp., use a series of strategies similar to those employed by closely related schistosomes (i.e. rapid development, stealth-like host-interfaces, and immunosuppression; {Wilson, 2009 #XXXXXX}), to avoid the immunosurveillance of their hosts {Leow, 2014 #XXXXXX}. This could certainly explain the presence of the tegument and/or mucus observed to cover the spines of *P. symplocos* (see Fig. 21, page 37 in {Nolan, 2006 #8092}).

Here, although we found fishes infected with between 7–25 adult worms (see Fig. 5), which were calculated to account for between 7–30% of the total estimated heart volume of *T. fucata*, there appears to be little visual impact on host health. This is despite *P. heinigerae* n. sp. also possessing small tegumental spines in incomplete lateral transverse rows along the entire length of the body and the adults

being wound extensively throughout the intertrabecular spaces of the ventricle. Despite the absence of evidence of pathogenic effects, we find it unlikely that such dramatic infection of such a key organ could be without significant effect on host health.

Conflict of interest

The authors declare they have no competing interests.

Acknowledgements

This study was funded by, and is a contribution from, the Australian node of the *CReefs* global research initiative (grant number: 209/29), a project generously sponsored by BHP Billiton in partnership with The Great Barrier Reef Foundation, the Australian Institute of Marine Science, the Australian Biological Resources Study, and the Alfred P. Sloan Foundation. *CReefs* is a field program of the Census of Marine Life. We gratefully thank the staff of the Heron and Lizard Island Research Stations for their support and hospitality during our stays.

References

[1] Orelis-Ribeiro R, Arias CR, Halanych KM, Cribb TH, Bullard SA. Diversity and ancestry of flatworms infecting blood of nontetrapod craniates "fishes". Adv Parasitol 2014;85:1–64.

[2] Orelis-Ribeiro R, Bullard SA. Blood flukes (Digenea: Aporocotylidae) infecting body cavity of South American catfishes (Siluriformes: Pimelodidae): two new species from rivers in Bolivia, Guyana and Peru with a re-assessment of *Plehniella* Szidat, 1951. Folia Parasitol (Praha) 2015;62:050.

[3] Santoro M, Cipriani P, Pankov P, Lawton SP. *Aporocotyle michaudi* n. sp. (Digenea: Aporocotylidae) from the emerald rock cod, *Trematomus bernacchii* (Teleostei: Perciformes) in Antarctica. Parasitol Int 2015;64:324–9.

[4] Ogawa K, Akiyama K, Grabner D. *Paradeontacylix buri* n. sp. (Trematoda: Aporocotylidae) from *Seriola quinqueradiata* cultured in Japan with a description of unidentified *Paradeontacylix* sp. from *S. lalandi*. Fish Pathol 2015;50:183–91.

[5] Orelis-Ribeiro R, Bullard SA. Two new genera of fish blood flukes (Digenea: Aporocotylidae) from catfishes in the Peruvian Amazon. J Parasitol 2016:In-Press.

[6] Nolan MJ, Cribb TH. An exceptionally rich complex of Sanguinicolidae von Graff, 1907 (Platyhelminthes: Trematoda) from Siganidae, Labridae and Mullidae (Teleostei: Perciformes) from the Indo-West Pacific Region. Zootaxa 2006;1218:1–80.

[7] Nolan MJ, Cribb TH. *Cardicola* Short, 1953 and *Braya* n. gen. (Digenea: Sanguinicolidae) from five families of tropical Indo-Pacific fishes. Zootaxa 2006;1265:1–80.

[8] Nolan MJ, Miller TL, Cutmore SC, Cantacessi C, Cribb TH. *Cardicola beveridgei* n. sp. (Digenea: Aporocotylidae) from the mangrove jack, *Lutjanus argentimaculatus* (Perciformes: Lutjanidae), and *C. bullardi* n. sp. from the Australian spotted mackerel, *Scomberomorus munroi* (Perciformes: Scombridae), from the northern Great Barrier Reef. Parasitol Int 2014;63:735–45.

[9] Yong RQ-Y, Cutmore SC, Miller TL, Adlard RD, Cribb TH. The ghost of parasites past: eggs of the blood fluke *Cardicola chaetodontis* (Aporocotylidae) trapped in the heart and gills of butterflyfishes (Perciformes: Chaetodontidae) of the Great Barrier Reef. Parasitology 2013;140:1186–94.

[10] Overstreet RM, Køie M. *Pearsonellum corventum*, gen. et sp. nov. (Digenea: Sanguinicolidae), in serranid fishes from the Capricornia section of the Great Barrier Reef. Aust J Zool 1989;37:71–9.

[11] Nolan MJ, Cribb TH. Two new blood flukes (Digenea: Sanguinicolidae) from Epinephelinae (Perciformes: Serranidae) of the Pacific Ocean. Parasitol Int 2004;53:327–35.

[12] Cribb TH, Bray RA. Trematodes of fishes: a test case for predictions of parasite diversity on the Great Barrier Reef. In: Greenwood JG, Hall NJ, editors. Proceedings of the Australian Coral Reef Society 75th Anniversary Conference, Heron Island October 1997. Brisbane: School of Marine Science; 1998. p. 43–56.

[13] Yong RQ-Y, Cribb TH. *Rhaphidotrema kiatkiongi*, a new genus and species of blood fluke (Digenea: Aporocotylidae) from *Arothron hispidus* (Osteichthyes: Tetraodontidae) from the Great Barrier Reef, Australia. Folia Parasitol 2011;58:273–7.
[14] Blasco-Costa I, Cutmore SC, Miller TL, Nolan MJ. Molecular approaches to trematode systematics: 'best practice' and implications for future study. Syst Parasitol 2016;93:295–306.
[15] Cutmore SC, Miller TL, Curran SS, Bennett MB, Cribb TH. Phylogenetic relationships of the Gorgoderidae (Platyhelminthes: Trematoda), including the proposal of a new subfamily (Degeneriinae n. subfam.). Parasitol Res 2013;112:3063–74.
[16] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–7.
[17] Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 2004;5:113.
[18] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–8.
[19] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.
[20] Heiniger H, Cribb TH, Adlard RD. Intra-specific variation of *Kudoa* spp. (Myxosporea: Multivalvulida) from apogonid fishes (Perciformes), including the description of two new species, *K. cheilodipteri* n. sp. and *K. cookii* n. sp., from Australian waters. Syst Parasitol 2013;84:193–215.
[21] Heiniger H, Adlard RD. Host specificity and local infection dynamics of *Kudoa leptacanthae* n. sp. (Multivalvulida: Kudoidae) from the pericardial cavity of two *Zoramia* spp. (Perciformes: Apogonidae) at Lizard Island lagoon, Queensland, Australia. Parasitol Int 2012;61:697–706.
[22] Leis JM, Trnski T, Dufour V, Harmelin-Vivien M, Renon J-P, Galzin R. Local completion of the pelagic larval stage of coastal fishes in coral-reef lagoons of the Society and Tuamotu Islands. Coral Reefs 2003;22:271–90.
[23] Atema J, Kingsford MJ, Gerlach G. Larval reef fish could use odour for detection, retention and orientation to reefs. Mar Ecol Prog Ser 2002;241:151–60.
[24] Gerlach G, Atema J, Kingsford MJ, Black KP, Miller-Sims V. Smelling home can prevent dispersal of reef fish larvae. Proc Natl Acad Sci 2007;104:858–63.
[25] Stobutzki IC, Bellwood DR. Nocturnal orientation to reefs by late pelagic stage coral reef fishes. Coral Reefs 1998;17:103–10.
[26] Gardiner NM, Jones GP. Synergistic effect of habitat preference and gregarious behaviour on habitat use in coral reef cardinalfish. Coral Reefs 2010;29:845–56.
[27] Gardiner NM, Jones GP. Habitat specialisation and overlap in a guild of coral reef cardinalfishes (Apogonidae). Mar Ecol Prog Ser 2005;305:163–75.
[28] Kuwamura T. Social and reproductive behaviour of three mouthbrooding cardinalfishes, *Apogon doederleini*, *A. niger* and *A. notatus*. Env Biol Fish 1985;13:17–24.

[29] Shirakashi S, Tani K, Ishimaru K, Shin SP, Honryo T, Uchida H, Ogawa K. Discovery of intermediate hosts for two species of blood flukes *Cardicola orientalis* and *Cardicola forsteri* (Trematoda: Aporocotylidae) infecting Pacific bluefin tuna in Japan. Parasitol Int 2016;65:128–36.

[30] Sugihara Y, Yamada T, Tamaki A, Yamanishi R, Kanai K. Larval stages of the bluefin tuna blood fluke *Cardicola opisthorchis* (Trematoda: Aporocotylidae) found from *Terebella* sp. (Polychaeta: Terebellidae). Parasitol Int 2014;63:295–9.

[31] Cribb TH, Adlard RD, Hayward CJ, Bott NJ, Ellis D, Evans D, Nowak BF. The life cycle of *Cardicola forsteri* (Trematoda: Aporocotylidae), a pathogen of ranched southern bluefin tuna, *Thunnus maccoyi*. Int J Parasitol 2011;41:861–70.

[32] Køie M. The redia, cercaria and early stages of *Aporocotyle simplex* Odhner, 1900 (Sanguinicolidae) - a digenetic trematode which has a polychaete annelid as the only intermediate host. Ophelia 1982;21:115–45.

[33] Betancur RR, Broughton RE, Wiley EO, Carpenter K, Lopez JA, Li C, Holcroft NI, Arcila D, Sanciangco M, Cureton Ii JC, Zhang F, Buser T, Campbell MA, Ballesteros JA, Roa-Varon A, Willis S, Borden WC, Rowley T, Reneau PC, Hough DJ, Lu G, Grande T, Arratia G, Orti G. The tree of life and a new classification of bony fishes. PLoS Curr 2013;5.

[34] Trieu N, Cutmore SC, Miller TL, Cribb TH. A species pair of *Bivesicula* Yamaguti, 1934 (Trematoda: Bivesiculidae) in unrelated Great Barrier Reef fishes: implications for the basis of speciation in coral reef fish trematodes. Syst Parasitol 2015;91:231–9.

[35] Evans WA. Growth, mortality, and hematology of cutthroat trout experimentally infected with the blood fluke *Sanguinicola klamathensis*. J Wildl Dis 1974;10:341–6.

[36] Evans WA. The histopathology of cutthroat trout experimentally infected with the blood fluke *Sanguinicola klamathensis*. J Wildl Dis 1974;10:243–8.

[37] Kirk RS, Lewis JW. Migration and development of the blood fluke *Sanguinicola inermis* Plehn, 1905 (Trematoda: Sanguinicolidae) in carp, *Cyprinus carpio* L. Parasitology 1996;113:279–85.

[38] Kirk RS, Lewis JW. Histopathology of *Sanguinicola inermis* infection in carp, *Cyprinus carpio*. J Helminth 1998;72:33–8.

[39] Ogawa K, Hattori K, Hatai K, Kubota S. Histopathology of cultured marine fish, *Seriola purpurascens* (Carangidae) infected with *Paradeontacylix* spp. (Trematoda: Sanguinicolidae) in its vascular system. Fish Pathol 1989;24:75–81.

[40] Ogawa K, Fukudome M. Mass mortality caused by blood fluke (*Paradeontacylix*) among amberjack (*Seriola dumerili*) imported to Japan. Fish Pathol 1994;29:265–9.

[41] Richards DT, Hoole D, Lewis JW, Ewens E, Arme C. Ultrastructural observations on the cellular response of carp, *Cyprinus carpio* L., to eggs of the blood fluke *Sanguinicola inermis* Plehn, 1905 (Trematoda: Sanguinicolidae). J Fish Dis 1994;17:439–46.

[42] Richards DT, Hoole D, Lewis JW, Ewens E, Arme C. Changes in the cellular composition of the spleen and pronephros of carp *Cyprinus carpio* infected with the blood fluke *Sanguinicola inermis* (Trematoda: Sanguinicolidae). Dis Aquat Organ 1994;19:173–9.

[43] Shirakashi S, Kishimoto Y, Kinami R, Katano H, Ishimaru K, Murata O, Itoh N, Ogawa K. Morphology and distribution of blood fluke eggs and associated pathology in the gills of cultured Pacific bluefin tuna, *Thunnus orientalis*. Parasitol Int 2012;61:242–9.

[44] Overstreet RM, Thulin J. Response by *Plectropomus leopardus* and other serranid fishes to *Pearsonellum corventum* (Digenea: Sanguinicolidae), including melanomacrophage centres in the heart. Aust J Zool 1989;37:129–42.

[45] Herbert BW, Shaharom-Harrison FM, Anderson IG. Histopathology of cultured sea bass (*Lates calcarifer*) (Centropomidae) infected with *Cruoricola lates* (Trematoda: Sanguinicolidae) from Pulau Ketam, Malaysia. Int J Parasitol 1995;25:3–13.

[46] Herbert BW, Shaharom-Harrison FM. A new blood fluke, *Parasanguinicola vastispina* gen. nov., sp. nov. (Trematoda: Sanguinicolidae), from sea bass *Lates calcarifer* (Centropomidae), cultured in Malaysia. Parasitol Res 1995;81:349–54.

[47] Alama-Bermejo G, Montero FE, Raga JA, Holzer AS. *Skoulekia meningialis* n. gen., n. sp. (Digenea: Aporocotylidae Odhner, 1912) a parasite surrounding the brain of the Mediterranean common two-banded seabream *Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817) (Teleostei: Sparidae): description, molecular phylogeny, habitat and pathology. Parasitol Int 2011;60:34–44.

505 **Table 1**

506 Numbers of specimens examined for 22 apogonid species collected from off Heron
507 Island and Lizard Island on the Great Barrier Reef, and on Ningaloo Reef off Western
508 Australia. The infection of *T. fucata* by *P. heinigerae* n. sp. is presented as number of
509 fish infected/number of fish sampled.
510

Genus Species	Heron Island	Lizard Island	Ningaloo Reef	Totals
Archamia		**1**		**1**
bleekeri (Günther)		1		1
Cercamia		**1**		**1**
eremia (Allen)		1		1
Cheilodipterus	**37**	**65**	**1**	**103**
artus Smith		12		12
intermedius Gon	5*	19	1	25
macrodon (Lacepède)	2*			2
quinquelineatus Cuvier	30*	34		64
Nectamia	**31**	**21**	**1**	**53**
fusca (Quoy & Gaimard)	31*	21		52
savayensis (Günther)			1	1
Ostorhinchus	**52**	**46**	**56**	**154**
angustatus (Smith & Radcliffe)	1		1	2
aureus (Lacepède)			14	14
compressus (Smith & Radcliffe)		5		5
cookii (Macleay)	34	1	1	36
cyanosoma (Bleeker)		4	24	28
doederleini (Jordan & Snyder)	17*	2		19
properuptus (Whitley)		5		5
rubrimacula (Randall & Kulbicki)		29		29
rueppellii (Günther)			16	16
Pristiapogon		**2**		**2**
exostigma (Jordan & Starks)		2		2
Rhabdamia		**10**		**10**
gracilis (Bleeker)		10		10
Taeniamia	**20**	**106**		**126**
fucata (Cantor)	19/20	27		47
zosterophora (Bleeker)		79		79
Zoramia		**274**		**274**
leptacantha (Bleeker)		274		274
Totals	140	526	58	724

511

512 * Species of apogonid sampled from the same Heron Island lagoon patch reefs that
513 infected individuals of *T. fucata* were collected from.
514

Table 2

Morphometric comparison of *Phthinomita heinigerae* n. sp. with the 11 recognised species of *Phthinomita* Nolan & Cribb, 2006. Shading indicates morphometric distinctions between *P. heinigerae* n. sp. and described species. Percentages (%) are based on total body length. Measurement of morphological characters from the anterior or posterior end of worms reflects the distance from the extremities of each feature.

	Character																		
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	References
P. heinigerae n. sp.	2977–3539 (3249)	16.5–22.4	22–25	1–2	19–32	12.4–37.9	6.2–8.2	28–37	83–99	8.3–13.0	1.0–1.6	3–4	35–56	6–7	1.3–1.9	2–3	40–60	11–13	Present study
P. symplocos (type-species)	3536–4858 (4217)	20.6–31.8	20–30	1–2	20–28	12.8–34.8	5.0–9.2	17–30	59–96	3.5–8.1	1.1–3.1	4–7	30–74	7–12	1.0–1.8	1–2	43–65	13–18	[6]
P. adlardi	4353–6294 (5394)	23.8–45.3	13–22	0–3	15–28	7.8–75.0	12.0–21.7	37–59	77–96	12.6–38.2	1.6–3.6	1–4	9–62	6–8	1.0–2.2	2–3	52–76	11–14	[6]
P. brooksi	4078–7843 (5207)	21.4–40.7	23–31	0–2	16–50	12.4–94.0	3.8–14.1	12–24	22–92	2.4–6.8	1.2–3.3	3–6	35–78	5–10	1.3–7.0	1–2	40–71	11–22	[6]
P. hallae	3070–3950 (3507)	24.5–32.8	20–35	?	?	?	10.1–21.7	35–37	48–95	7.6–9.9	1.8–2.5	3–5	37–66	6–10	0.6–1.3	1–2	40–57	9–15	[6]
P. ingramae	2317–2983 (2645)	15.6–27.4	18–32	1–3	21–46	7.8–24.0	5.4–11.3	23–47	44–100	4.9–10.7	1.1–2.4	3–6	46–71	7–10	0.9–2.0	2–3	46–72	13–21	[6]
P. jonesi	2060–5329 (3674)	23.6–75.0	16–40	1–2	15–35	8.1–71.8	5.3–19.8	18–40	71–100	3.5–22.4	1.1–6.3	1–5	29–70	6–12	0.9–1.7	1–2	45–95	9–20	[6]
P. littlewoodi	2993–4133 (3465)	26.3–59.7	24–42	1–2	14–29	8.5–26.7	4.9–15.5	16–31	62–97	4.4–12.4	1.7–3.3	2–4	30–67	6–10	0.9–2.1	1–2	46–80	11–19	[6]
P. robertsthomsoni	3784–5706 (4851)	19.8–34.7	9–36	1–2	13–31	10.0–37.5	9.0–22.9	38–62	65–94	14.8–31.3	1.2–3.3	2–3	15–46	5–10	1.2–1.4	2–4	30–71	10–18	[6]
P. sasali	3765–4017 (3863)	24.7–29.7	25–26	1–2	23–28	12.9–21.0	5.2–10.2	20–29	70–94	3.3–9.6	1.2–2.8	3–4	2	7–10	2.0–2.6	2	46–65	18–20	[6]
P. munozae	2714–6094 (5210)	31.8–41.8	21–28	1–2	30–41	13.1–37.5	11.2–21.2	27–44	67–100	11.7–25.0	1.8–5.0	2–3	11–43	5–7	1.1–2.1	1–2	35–85.0	11–15	[6]
P. poulini	2350–4269 (3451)	21.0–35.6	20–27	1–2	26–34	14.2–39.5	8.6–15.2	35–40	84–94	4.9–13.6	1.6–3.8	3–8	32–64	7–15	1.6–12.8	1–3	50–75	14–26	[6]

Character legend: 1) body length; 2) body length/width; 3) oesophagus %; 4) anterior caeca length %; 5) posterior caeca length %; 6) posterior caeca/anterior caeca; 7) anterior testis length/width; 8) anterior testis length %; 9) anterior testis width %; 10) anterior testis length/posterior testis length; 11) posterior testis length/width; 12) posterior testis length %; 13) posterior testis width %; 14) cirrus-sac position %; 15) cirrus-sac length/width; 16) ovary length %; 17) ovary width %; 18) ovary position (%)

List of Figures

Figs. 1–2. *Phthinomita heinigerae* n. sp. from *T. fucata* from off Heron Island. 1. Holotype, adult, whole mount, lateral view. 2. Holotype, female terminal genitalia, lateral view. Abbreviations: GP, female genital pore; MG, Mehlis' gland; OD, oviduct; Oö, oötype; Ov, ovary; T, testis; U, uterus; VD, vitelline duct; VF, vitelline follicles. Scale–bars: 1, 500 μm; 2, 250 μm.

Fig. 3. The genetic relationships among species of *Phthinomita* inferred by minimum evolution analysis of the complete ITS2 rDNA dataset. The sequence from the present study is indicated in bold. Bootstrap support is indicated for all major nodes.

Figs. 4–5. The heart of *T. fucata*. 4. Dissected heart illustrating the intensity of a *P. heinigerae* n. sp. infection in a single host fish. 5. Longitudinal section illustrating *P. heinigerae* n. sp. occupying the intertrabecular spaces and lumen of the ventricle. Scale–bar: 4, 200 μm.