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DNA sequencing demonstrates the importance of jellyfish in life cycles of lepocreadiid trematodes

J.G. Browne[1,2,*], K.A. Pitt[1] and T.H. Cribb[3]

[1]School of Environment and Science and Australian Rivers Institute, Griffith University, Gold Coast Campus, Gold Coast, Queensland 4222, Australia; [2]Museums Victoria, GPO Box 666, Melbourne, Victoria 3001, Australia and [3]School of Biological Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia

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*Current address: Department of Water and Environmental Regulation, 72 Duchess Street, Busselton, Western Australia 6280, Australia.

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Author for correspondence:
J.G. Browne,
E-mail: joanna.browne@dwer.wa.gov.au

Abstract

Sequence data were combined with morphological analyses to identify two lepocreadiid trematode species from jellyfishes and fishes. Three species of jellyfish were captured within Port Phillip Bay, Australia, and three species of fish that feed on jellyfish were obtained from Moreton Bay (Queensland) and Port Phillip Bay and Portland (Victoria). The digeneans were distributed throughout most parts of the jellyfish. *Opechona* cf. *kahawai* Bray & Cribb, 2003 parasitized the scyphozoan jellyfish *Aequorea eurodina* and the scombrid fish *Scomber australasicus*. *Cephalolepidapedon warehou* Bray & Cribb, 2003 parasitized the scyphozoans *Pseudorhiza haeckeli* and *Cyanea annaskala*, and the centrolophid fishes *Seriolella brama* and *Seriolella punctata*. Intensities ranged from four to 96 in the jellyfish, and one to 30 in the fish. For both trematode species, internal transcribed spacer 2 of ribosomal DNA sequences from mature adults in the fishes matched those from metacercariae from the jellyfish. This is the first record of larval stages of *C. warehou* and *O.* cf. *kahawai*, and the first use of DNA sequencing to identify digenean trematode metacercariae from jellyfish. Three new host records are reported for *C. warehou* and two for *O.* cf. *kahawai*.

Introduction

Elucidating the life cycles of parasites with complex life histories is useful for understanding the pathology of infections (Catalano *et al.*, 2011) and to elucidate trophic linkages (Williams *et al.*, 1992), migrations (e.g. Carballo *et al.*, 2012) and environmental changes (Palm *et al.*, 2011). Although the rate at which new parasites are being identified is accelerating, elucidation of life cycles has waned (Blasco-Costa & Poulin, 2017).

Until recently, jellyfish have been considered trophic dead ends (i.e. rarely eaten; Hays *et al.*, 2018), and so their potential role as vectors for transferring intermediate stages of digenean parasites to vertebrate hosts was seldom considered. New technologies, including DNA metabarcoding, animal-borne cameras and stable isotope analysis, however, reveal that a surprising diversity of vertebrates prey on jellyfish, including flying seabirds, penguins, turtles and fishes (Hays *et al.*, 2018). Indeed, jellyfish are now recognized to be consumed by at least 124 species of fishes (Arai, 1988; Purcell & Arai, 2001; Arai, 2005; Pauly et al., 2009). Digeneans are often more prevalent in jellyfish than in other zooplankton intermediate hosts such as copepods (Marcogliese, 1995) and thus may be important for transferring parasites to definitive hosts.

Jellyfish have been reported as intermediate hosts for the metacercariae of at least 17 digenean species and nearly 70 species of jellyfish (medusae, siphonophores and ctenophores) host digeneans (Browne, 2015). The metacercariae found in jellyfish, however, are difficult to identify, which has hampered our ability to identify them and their definitive hosts. Life cycles of digeneans infecting jellyfish have thus been primarily elucidated by feeding experiments (e.g. Stunkard, 1980b). Such studies have shown that some lepocreadiid cercariae emerge from their first intermediate mollusc host, swim in the water column, and directly penetrate jellyfish, which are the second intermediate host, where they form metacercariae (Stunkard, 1969, 1972, 1980a, b; Køie, 1975). When the jellyfish are eaten by fish, the metacercariae develop into sexual adult digeneans (Stunkard, 1969, 1980a, b). Lepocreadiid genera that use jellyfish as intermediate hosts include species of *Opechona* Looss, 1907 (e.g. Køie, 1975; Stunkard, 1980b; Martorelli, 2001), *Cephalolepidapedon* Yamaguti, 1970 (e.g. Ohtsuka *et al.*, 2010), *Lepidapedon* Stafford, 1904 (Køie, 1985), *Lepocreadium* Stossich, 1904 (e.g. Stunkard, 1972, 1980a; Bray & Cribb, 1996) and *Lepotrema* Ozaki, 1932 (e.g. Ohtsuka *et al.*, 2010).

Analysis of ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) enables the identification of digenean species (Nolan & Cribb, 2005; Blasco-Costa *et al.*, 2016). Sequences from unidentified metacercariae can be matched to those of adults and, coupled with morphological data and knowledge of host biology, allow insights into the life cycles of the parasites (Cribb *et al.*, 1998). Molecular identification has not been applied to metacercariae found in jellyfish,

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but this approach may reveal jellyfish to be significant intermediate hosts of digeneans that infect marine vertebrates.

This study utilized the ITS2 of rDNA. This region is highly variable yet relatively conserved within platyhelminth species, and, thus, is useful for identifying larval forms (Adlard *et al.*, 1993; Tandon *et al.*, 2007; Skov, 2009). The aims of this study were to identify metacercariae from jellyfish by comparing ITS2 sequences of the metacercariae from jellyfish to those of adult digeneans in fishes that predate upon jellyfish, and to thus investigate the role of jellyfish in the life cycles of digeneans.

Materials and methods

Collection of specimens

Jellyfish were sampled opportunistically from within Port Phillip Bay (38°05′17″–38°17′1″S, 144°36′54″–144°43′58″E), Australia, between September 2009 and February 2012. Three species were collected: three *Cyanea annaskala* Von Lendenfeld, 1882 (Scyphozoa; Semaeostomeae); seven *Pseudorhiza haeckeli* Haacke, 1884 (Scyphozoa; Rhizostomeae) and six *Aequorea eurodina* Péron & Lesueur, 1810 (Hydrozoa; Leptothecata; table 1). *Cyanea annaskala* and *P. haeckeli* were collected using a dip net from a boat or by zip-lock bag while snorkelling. *Aequorea eurodina* were collected from the shoreline after having been washed ashore. Although these medusae were dead, the digenean metacercariae in them were alive when collected. Jellyfish were returned to the laboratory (alive if possible) and their diameters were measured. Medusae were examined under a Leica Wild M8 stereomicroscope (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia) and digeneans were removed. The location of digeneans within the jellyfish (bell, tentacles, oral arms (*P. haeckeli* only), oral pillar/disk and gut) was recorded.

Three fish species known to feed upon jellyfish were obtained between June 2009 and April 2011 (table 1): *Scomber australasicus* Cuvier, 1832; *Seriolella brama* (Günther, 1860); and *Seriolella punctata* (Forster, 1801). Five *S. brama* were trawled within Port Phillip Bay (37°58′30″–38°14′34″S, 144°46′01″–144°52′38″E); three *S. punctata* were trawled near Portland, Victoria (within the vicinity of 38°20′S, 141°36′E) and two *S. australasicus* individuals were caught in Moreton Bay, Queensland (27°07′54.28″S, 153°21′10.63″E). Although *S. australasicus* were sampled at a location distant from that of the jellyfish, their distribution overlaps with that of *A. eurodina* and so was considered worthy of investigation.

Fish were refrigerated or stored on ice (*S. punctata* and *S. brama*) or frozen (*S. australasicus*), and had been dead for 10–48 h before dissection. The total length of each fish was measured, and the digestive tract was removed and separated into stomach, pyloric caeca and intestine. Each section was examined for digeneans using a stereomicroscope, then shaken in a saline solution (one part seawater to three parts tap water) before re-examination (following Cribb & Bray, 2010).

Digeneans were removed from the jellyfish or fish/gut washes with a pipette and fixed in near-boiling seawater solution (Cribb & Bray, 2010). They were assigned to a morphotype and preserved in 10% formalin for morphological analysis or 96% absolute alcohol for molecular sequencing. Prevalence and mean intensity of each morphotype were calculated according to Bush *et al.* (1997).

Morphological analyses

Prior to DNA extraction, ethanol-preserved samples were examined in fresh water on a concave slide using an Olympus BX50 compound microscope (Olympus, Notting Hill, VIC, Australia). Digeneans were photographed using a QImaging Go-21 CMOS camera (Adept Turnkey, Coburg, VIC, Australia) mounted on the microscope and measured using an ocular micrometre. The morphological characters measured were as follows: body length, body width, length of forebody, oral sucker width, oral sucker length, ventral sucker width and ventral sucker length. The forebody was measured as the distance between the anterior extremity of the body and the anterior margin of the ventral sucker. The prepharynx was measured as the distance from distal end of oral sucker to proximal end of pharynx.

Digeneans preserved in formalin were rinsed in water and overstained with Mayer's haematoxylin. They were then rinsed in fresh water, destained with 1% hydrochloric acid and neutralized in 1% ammonium hydroxide solution (Miller & Cribb, 2007). The specimens were dehydrated through a series of ethanol solutions between 70 and 100%, cleared in methyl salicylate and mounted in Canada balsam. The digeneans were viewed using an Olympus BX50 compound microscope and drawn with a camera lucida. Images were digitized using a Wacom tablet and Adobe Illustrator.

Molecular analyses

Genomic DNA was isolated from single specimens using proteinase K and either the phenol/chloroform extraction procedure (Sambrook *et al.*, 2001) or a QIAamp® DNA Mini Kit (QIAGEN, Hilden, NW, Germany). Due to the small size of the trematodes (some <200 µm), the following modifications were made. Prior to extraction, each trematode was pipetted into a vial with a minimal amount of ethanol. The vial lids were left open until the ethanol had evaporated (removing the risk of losing the digenean when aspirating off solution). TE buffer and proteinase K were added, the solution was centrifuged and vortexed and then placed overnight in a rotating incubator. The ITS2 region was amplified using the forward primer '3S' (5′-GGTACCGGTGG ATCACGTGGCTAGTG-3) (3′ end of 5.8S rDNA) (Bowles *et al.*, 1993) and the reverse primer 'ITS2.2' (5′-CCTGGTTAG TTTCTTTTCCTCCGC-3′) (5′ end of 28S rDNA) (Cribb *et al.*, 1998). The polymerase chain reaction (PCR) reactions were carried out in 20 µl volumes, each with 4 µl of Hotstar Q solution (QIAGEN), 2 µl of 10× PCR reaction buffer, 0.8 µl of 10 mM deoxyribonucleotide triphosphate (dNTP), 0.75 µl of each primer (Invitrogen, Carlsbad, CA, USA) (10 µM), 0.25 µl of HotstarTaq (QIAGEN), 6.45 µl of nuclease-free water and 5 µl of template DNA. Amplification included an initial step of 95°C for 15 min followed by 35 cycles of denaturation at 96°C for 45 s, annealing at 48°C for 30 s and extension at 72°C for 45 s followed by a final extension step of 72°C for 4 min and a holding temperature of 15°C. Reamplification was necessary for the metacercariae samples (most likely due to the small size of each specimen). For these reactions, 1 µl of PCR product was used instead of 5 µl of DNA template, and 10.45 µl of nuclease-free water. Positive and negative controls were run for all amplifications. The number of sequences obtained for the digenean species from each host was *C. annaskala* ($n = 3$), *P. haeckeli* ($n = 4$), *A. eurodina* ($n = 1$), *Seriolella brama* ($n = 4$), *S. punctata* ($n = 1$) and *Scomber australasicus* ($n = 2$). The amplified DNA was purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) following the manufacturer's recommended protocol. The purified product was sequenced by Macrogen, South Korea.

Forward and reverse sequences were edited to produce a single sequence for each specimen using BioEdit version 7.0.9.0 (Hall,

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Table 1. Digenean species, species of hosts dissected, number of hosts (*n*), prevalence (P) of infection, mean intensity of digeneans and range, and size of host (bell diameter (BD) for jellyfish, total length (TL) for fish).

Digenean species	Host species	*n*	P (%)	Mean intensity ± SE (range)	BD/TL (cm)
Cephalolepidapedon warehou	Jellyfish				
	Cyanea annaskala	3	33	6 (6)	1.8–8.0
	Pseudorhiza haeckeli	7	71	24.0 ± 18.0 (4–96)	4.0–21.0
	Fish				
	Seriolella brama	5	100	13.3[a] ± 6.0 (5–25)	3.2–26.0
	Seriolella punctata	3	67	3.0 ± 2 (1–5)	43.0–48.0
Opechona cf. *kahawai*	Jellyfish				
	Aequorea eurodina	6	100	15.3 ± 4.4 (5–30)	2.2–3.5
	Fish				
	Scomber australasicus	2	100	7.5 ± 3.5 (4–11)	22.0–22.5

[a]Mean intensity only calculated from three fish specimens. SE, standard error.

1999). As the sequences obtained included the entire ITS2 region and sections of the adjoining 5.8S and 28S, the ITS2 sequence was isolated using the annotation tool of the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) using the default parameters (Keller *et al.*, 2009). Sequences were aligned in MEGA 5.05 (Tamura *et al.*, 2011) using MUSCLE with the defaults selected, except maximum iterations, which were changed to ten. Alignment was checked by eye in Mesquite (Maddison & Maddison, 2011) and the ends were trimmed to match the shortest sequence. Distance matrices were constructed using MEGA 5.05 (Tamura *et al.*, 2011) to calculate the number of base differences per sequence. Pairwise deletion was selected to remove ambiguous positions. A basic local alignment search tool (BLAST) search was undertaken in GenBank to look for similar sequences. Sequences were lodged with GenBank for each parasite host combination, including all sequence variants.

Results

All adult trematodes from *S. punctata* and *S. brama* were identified as *Cephalolepidapedon warehou* Bray & Cribb, 2003 (family Lepocreadiidae; table 1) by comparison with the original description. Adult trematodes from *S. australasicus* (table 1) were in poor condition for morphological study but were consistent with the genus *Opechona* (elongate body, infundibuliform oral sucker, pseudo-oesophagus present). There are only two species of *Opechona* known in Australian waters, *Opechona austrobacillaris* Bray & Cribb, 1998 and *O. kahawai* Bray & Cribb, 2003. Sequences from the present species differ from those of *O. austrobacillaris* reported by Bray *et al.* (2018) by 20 bases of ITS2 rDNA, but unfortunately no ITS2 sequences are available to corroborate the identification of *O. kahawai*. *Opechona kahawai* has previously been reported from a species of *Arripis* (Arripidae, type host) and from *Seriola lalandi* Valenciennes, 1833 (Carangidae) by Hutson *et al.* (2007). Infection of this species in *S. australasicus* appears plausible, but the taxonomy of this difficult genus certainly requires further work, so we take the conservative position of identifying it as *Opechona* cf. *kahawai*.

Digenean metacercariae from the scyphozoan jellyfish *P. haeckeli* and *C. annaskala* were identified as *C. warehou*, inferred from ITS2 sequences identical to those from adult digeneans (table 2). Morphological features (fig. 1a) were broadly consistent with those of adult *C. warehou* according to Bray & Cribb (2003), although the metacercariae clearly undergo extensive development in the definitive host. Metacercariae from the hydrozoan jellyfish *A. eurodina* were identified as *O.* cf. *kahawai* using ITS2 sequences identical to those of adult *O.* cf. *kahawai* (table 2 and fig. 1b); again, the metacercariae clearly develop extensively in the definitive host.

Molecular results

Sequences of *C. warehou* from seven adult digeneans from *S. brama* and *S. punctata* were identical to those from seven metacercariae from *P. haeckeli* and *C. annaskala* (table 2). All sequences contained complete ITS2 sequences of 290 bases, except two shortened sequences from *C. annaskala* metacercariae. Apart from the missing ends, all sequences were identical except one for one specimen from *S. brama*, which differed by only one transition.

Sequences of two adult *O.* cf. *kahawai* from *S. australasicus* matched sequences from two metacercariae from two specimens of *A. eurodina* (table 2). All sequences contained complete ITS2 sequences of 293 bases. One sequence from *S. australasicus* was identical to one from *A. eurodina* and the other two sequences differed from these by one transition and three base pairs transitions (and by one transition to each other).

Morphological description of metacercariae

Cephalolepidapedon warehou metacercariae from jellyfish

Measurements are provided in table 3. Body elongate, rounded posteriorly (fig. 1a). Tegument spinose, with spines in regular rows in forebody, sparse or absent in hindbody. Pigment copious, scattered throughout parenchyma of forebody, to about posterior margin of ventral sucker. Oral sucker funnel-shaped, terminal. Ventral sucker slightly smaller than oral sucker, rounded, pre-equatorial on slight protuberance. Prepharynx long; pharynx distinct, oval; forebody long. Caeca terminate blindly. Testes two, rounded to oval and entire, in mid-hindbody.

The morphology of the metacercariae resembles that of the adult digeneans from their fish hosts in the distinct funnel-shaped sucker and the long prepharynx (table 3). However, the forebody

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Table 2. Digenean species, host, locations, replicate information and GenBank accession numbers for trematode sequences.

Digenean species	Host	Locality	No. of hosts from which digenean sequences obtained	No. of digeneans from which sequences obtained	Size (bp)	Accession numbers
Cephalolepidapedon warehou	*Cyanea annaskala*	Port Phillip Bay	1	3	218, 287, 290	MT773345
	Pseudorhiza haeckeli	Port Phillip Bay	3	4	290	MT773346
	Seriolella brama	Port Phillip Bay	4	6	290	MT773347, MT773348
	Seriolella punctata	Off Portland	1	1	290	MT773349
Opechona cf. *kahawai*	*Aequorea eurodina*	Port Phillip Bay	2	2	293	MT773351, MT773353
	Scomber australasicus	Moreton Bay	2	2	293	MT773350, MT773352

is proportionally longer in the metacercariae and the pigmentation is far heavier in the forebody.

Opechona *cf.* kahawai *metacercariae from jellyfish hosts*

Measurements are provided in table 3. Body elongate (fig. 1b). Tegumental spines minute, most obvious in forebody. Eye-spot pigment heavy in region from oral sucker to more than halfway to ventral sucker. Oral sucker large, infundibuliform; may be withdrawn into forebody, with wide aperture, terminal or slightly ventrally subterminal. Prepharynx, pharynx, oesophagus and pseudo-oesophagus obscured by pigment in forebody. Intestinal bifurcation in forebody (exact location unable to be seen due to heavy pigmentation). Forebody long. Ventral sucker rounded, smaller than oral sucker, in posterior half of body, slightly protuberant. Excretory pore terminal. No testes, seminal vesicle, ovaries or other reproductive organs visible.

Inability to discern the digestive system in these metacercariae reduced possible points of comparison with sexual adults from fishes. As for *C. warehou*, the pigmentation was far heavier than is reported in the adults and the forebody was proportionally longer. Adult digeneans from *S. australasicus* were unable to be measured due to deterioration related to freezing in the host fish.

Prevalence and intensities of infection

Opechona cf. *kahawai* occurred only within *A. eurodina*, whereas *Cephalolepidapedon warehou* occurred in both *P. haeckeli* and *Cyanea annaskala*. Prevalence of *O.* cf. *kahawai* in the jellyfish was 100%, whereas that of *C. warehou* ranged from 33 to 100% (table 1). The maximum intensity of *C. warehou* was higher than that of *O.* cf. *kahawai* (table 1). *Cephalolepidapedon warehou* occurred in different areas of its jellyfish hosts, whereas *O.* cf. *kahawai* occurred only in the bell of *A. eurodina* (table 4). The metacercariae had not obviously damaged the tissues of the jellyfishes, but they were easily dislodged with a pipette suggesting a 'softening' of the surrounding tissue.

Cephalolepidapedon warehou occurred in the intestines of *S. brama* and *S. punctata*. All specimens of *S. brama* were parasitized, unlike *S. punctata*. Intensity of *C. warehou* was also higher in *S. brama* (table 1). Mature and immature specimens of *C. warehou* were found in both fish species. *Opechona* cf. *kahawai* occurred in the intestines of both specimens of *Scomber australasicus*, with intensities of four and 11 (table 1). Mature and immature *O.* cf. *kahawai* were present.

Discussion

Digenean metacercariae recovered from three jellyfish species of Port Phillip Bay were identified as two species of lepocreadiid digeneans (*C. warehou* and *O.* cf. *kahawai*) using DNA sequencing. This is the first time DNA sequencing has been used to identify digenean metacercariae in jellyfish and highlights their role as potentially important intermediate hosts of digenean trematodes. Three new host records for *C. warehou* were recorded, and one new host was recorded for *O.* cf. *kahawai*, thus allowing inference of partial life cycles for two digenean species.

Cephalolepidapedon warehou

Our study has revealed several new aspects of the life cycle of *C. warehou*. Our observations of *C. warehou* parasitizing the scyphozoans *P. haeckeli* and *Cyanea annaskala* are the first records of *C. warehou* parasitizing jellyfish and the first time an intermediate host for *C. warehou* has been identified. Moreover, although *C. warehou* was described from *S. punctata* in Tasmania (Bray & Cribb, 2003), our study identifies *S. brama* as another definitive host.

The presence of a metacercaria in any intermediate host does not necessarily demonstrate the host is involved in transmission; dead ends are also possible. However, the use of jellyfish as intermediate hosts by *C. warehou* is consistent with the close association of *S. brama* and *S. punctata* with jellyfish. Juveniles of both species of fish aggregate under jellyfish in Tasmania (Last *et al.*, 1983) and *S. brama* associate with *Catostylus mosaicus*, *P. haeckeli* and *A. eurodina* in Port Phillip (Browne, pers. obs.). As adults, *S. brama* and S. *punctata* feed primarily upon pyrosomes, salps and hyperiid amphipods (Bulman *et al.*, 2001; Horn *et al.*, 2011). Unidentified jellyfish were also found in the stomach contents of *S. punctata* (Horn *et al.*, 2011). The high percentage of 'unknown' stomach contents recorded in both species of fish (Bulman *et al.*, 2001; Horn *et al.*, 2011) may well include jellyfish. Indeed, jellyfish are difficult to identify in gut contents (Arai, 1988; Arai *et al.*, 2003), particularly when stomachs are frozen or preserved (Bulman *et al.*, 2001; Horn *et al.*, 2011). For Atlantic Ocean warehou, *Seriolella porosa*, ctenophores

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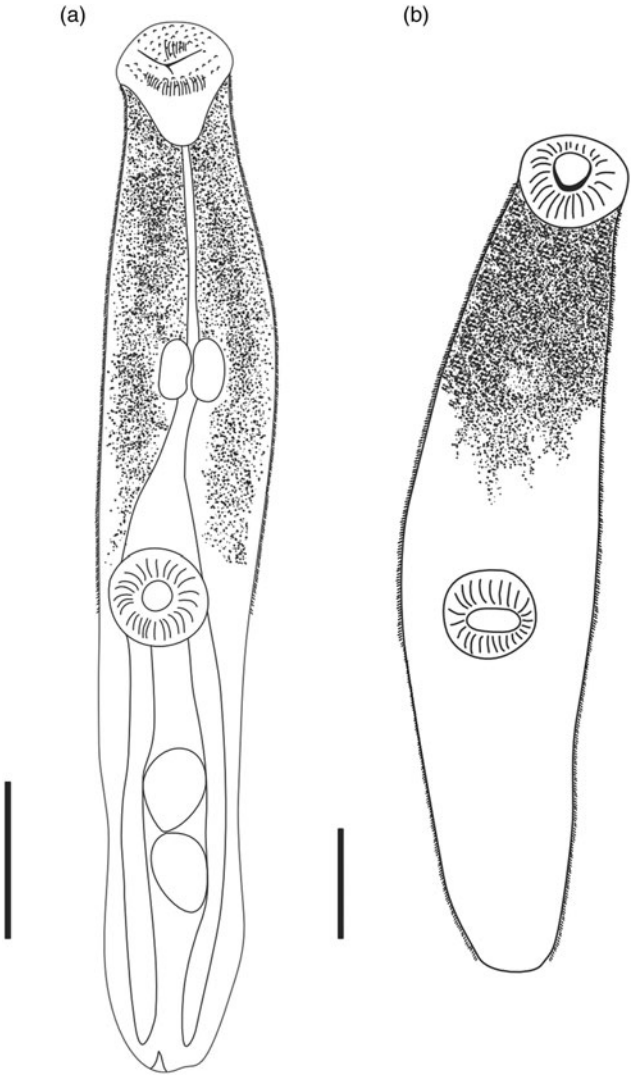

Fig. 1. (a) Ventral view of metacercaria of *Cephalolepidapedon warehou* from *Pseudorhiza haeckeli* from Port Phillip Bay (point of bifurcation of intestinal caeca anterior to ventral sucker not visible). Drawing a composite of four worms. (b) Ventral view of metacercaria of *Opechona* cf. *kahawai* from *Aequorea eurodina* from Port Phillip Bay. Drawing a composite of two worms. Scale bars: 100 µm.

comprise up to 78% of gut contents during summer (Mianzan *et al.*, 1996). As no records of digeneans parasitizing salps could be found in the literature, and many other lepocreadiid species use jellyfish and other gelatinous zooplankton as hosts (e.g. Stunkard, 1969, 1980a; Køie, 1975; Bray *et al.*, 2009), it is almost certain that jellyfish act as key intermediate hosts for *C. warehou*. The first intermediate host of *C. warehou* is unknown.

The use of jellyfish as hosts by species of *Cephalolepidapedon* is supported by knowledge of its only congener, *Cephalolepidapedon saba* Yamaguti, 1970. Metacercariae of *C. saba* have been recorded in the scyphozoan jellyfish *Aurelia aurita sensu lato* (Ohtsuka *et al.*, 2010; Kondo *et al.*, 2016), *Chysaora pacifica* and *Cyanea nozakii* (Kondo *et al.*, 2016). Adults and metacercariae of *C. saba* have been found in the intestines of the Japanese butterfish, *Psenopsis anomala* (Kondo *et al.*, 2016). This fish species associates with and feeds upon jellyfish (Masuda *et al.*, 2008; Ohtsuka *et al.*, 2009). *Cephalolepidapedon saba* has been identified in specimens of *C. pacifica*, and *C. nozakii* captured simultaneously with the associated fish *P. anomala*. Nematocysts in the intestines of the fish, supported by stable isotope studies, support the conclusion of Kondo *et al.* (2016) that transmission of *C. saba* to *P. anomala* occurs through medusivory. Adult *C. saba* have also been recorded from *Scomber japonicus* (Bray & Gibson, 1990; Bartoli & Bray, 2004) and *S. australasicus* (Korotaeva, 1974). These species are known to feed upon gelatinous zooplankton (Takano, 1954) and the congener *Scomber scombrus* feeds on the hydromedusa *Aglantha digitale* (Runge *et al.*, 1987).

Opechona cf. kahawai

Opechona kahawai is a little-studied parasite that has been recorded from only two host species, *Seriola lalandi* in Victoria (Hutson *et al.*, 2007) and *Arripis* (either *trutta* or *truttacea*) in Tasmania (Bray & Cribb, 2003). Therefore, our discovery of *O.* cf. *kahawai* parasitizing *Scomber australasicus* may be a new definitive host for *O. kahawai*. No intermediate hosts are known for *O. kahawai*, and our study represents the first record of *O.* cf. *kahawai* from an intermediate host, *A. eurodina*. The high degree of similarity between the DNA sequences of metacercariae from *A. eurodina* and adult digeneans from *S. australasicus* suggest that *A. eurodina* is an additional host for this species. Although one sequence from a digenean from *A. eurodina* exactly matched one sequence from a digenean from a *S. australasicus*, there were also differences of one and three base pairs relative to the other sequences. Intraspecific variation in ITS2 sequences is typically low, so it is possible that more than one species was present. However, the differences are more likely to be sequencing error, due to the difficulty of obtaining fresh digenean specimens from *S. australasicus* (the fish had been frozen and then defrosted for dissection). Further sequencing with a greater number of replicates from both hosts (and ideally also from the other known hosts of *O. kahawai*) are necessary to resolve this issue. While the life history of *O. kahawai* is poorly known, the life cycles of other species within the genus *Opechona* have been elucidated experimentally: *Opechona bacillaris* (Molin, 1859) (see Køie, 1975), *Opechona cablei* Stunkard, 1980 (see Stunkard, 1980b) and *Opechona pyriforme* (Linton, 1900) (see Stunkard, 1969). These species all use hydrozoan or scyphozoan medusae and/or ctenophores as intermediate hosts. Unencysted metacercariae of these and other *Opechona* species have been found in a wide range of gelatinous zooplankton, including hydrozoan jellyfish (Bray & Gibson, 1990; Gómez del Prado-Rosas *et al.*, 2000; Martorelli, 2001; Martell-Hernández *et al.*, 2011; Diaz Briz *et al.*, 2012), scyphozoan jellyfish (Bray & Gibson, 1990; Morandini *et al.*, 2005; Ohtsuka *et al.*, 2010; Nogueira Júnior *et al.*, 2014; Kondo *et al.*, 2016) and ctenophores (Yip, 1984; Martorelli, 2001; Morandini *et al.*, 2005).

In addition to gelatinous zooplankton, metacercariae of *Opechona* species are recorded from planktonic polychaetes (Reimer *et al.*, 1971), chaetognaths (Lebour, 1917; Reimer *et al.*, 1971; Køie, 1975; Øresland & Bray, 2005), a pelagic heteropod mollusc (Morales-Ávila *et al.*, 2018) and free in the plankton (Nicoll, 1910; Franc, 1951).

This study is the second report of an *Opechona* species from *S. australasicus*, the previous being *O. bacillaris* from the Great Australian Bight (Korotaeva, 1974). High prevalences (45–100%) of *O. bacillaris* were found in *S. australasicus* (45.2% of 42 fish) (Korotaeva, 1974). It is quite likely that the *O. bacillaris* identified by Korotaeva were, in fact, *O. austrobacillaris* or *O. kahawai*/*O.* cf. *kahawai*, as the species are morphologically similar (Bray & Cribb, 1998) and those species had not yet been described in 1974.

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Table 3. Measurements of digeneans found in jellyfish and fish.

Digenean species	*Cephalolepidapedon warehou*					*Opechona* cf. *kahawai*	
Host species	*Pseudorhiza haeckeli* (formalin)	*Pseudorhiza haeckeli* (ethanol)	*Cyanea annaskala* (ethanol)	*Seriolella brama* (ethanol)	*Seriolella brama* (formalin)	*Aequorea* sp. (formalin)	*Aequorea* sp. (ethanol)
Length	580–660	540–710	660–720	580–730	490–790	500	300–500
	613 ± 41.6	635 ± 47.7	693 ± 30.6	657 ± 75.1	638 ± 123		409 ± 71.8
	3	12	3	3	4	1	7
Width	60–80	70–110	90–110	80–150	50–120	90	58–100
	70 ± 10	85 ± 11.7	100 ± 10	107 ± 37.9	85 ± 28.9		82.9 ± 16.3
	3	12	3	3	4	1	7
Forebody length	260–310	250–340	320–340	250–330	230–310	270	170–270
	283 ± 25.2	301 ± 25.8	330 ± 10	287 ± 40.4	275 ± 34.2		214 ± 42.4
	3	12	3	3	4	1	7
Oral sucker width	70	60–80	70–80	50–100	70	50	35–60
	70	70.9 ± 5.39	76.7 ± 5.77	76.7 ± 25.2	70		47.9 ± 8.09
	1	11	3	3	1	1	7
Oral sucker length	65–70	50–75	60–70	55–70	60–80	40	35–50
	68.3 ± 2.89	62.1 ± 8.11	66.7 ± 5.77	61.7 ± 7.64	72.5 ± 9.57		39.3 ± 5.35
	3	12	3	3	4	1	7
Ventral sucker width	60	50–75	60–70	50–80	75	40	30–50
	60	61.4 ± 7.78	63.3 ± 5.77	63.3 ± 15.3	75		41.1 ± 9.08
	1	11	3	3	1	1	7
Ventral sucker length	50–60	55–70	60–60	60–70	50–70	45	30–50
	53.3 ± 5.77	61.3 ± 4.83	0	63.3 ± 5.77	63.8 ± 9.46		41.7 ± 7.45
	3	12	3	3	4	1	7
Length/width	7.5–9.67	6.18–9.57	6.55–7.78	4.87–7.33	5.33–9.8	5.56	4.22–5.67
	8.87 ± 1.19	7.58 ± 1.04	6.97 ± 0.70	6.48 ± 1.4	7.95 ± 1.91		4.98 ± 0.56
	3	12	3	3	4	1	7
Forebody as % of body length	42.4–53.4	44.4–50	45.7–51.5	42.4–45.2	39.2–46.9	54	44.2–56.7
	46.4 ± 6.12	47.4 ± 1.53	47.7 ± 3.32	43.6 ± 1.45	43.6 ± 3.35		52.5 ± 4.79
	3	12	3	3	4	1	7

Sucker width ratio	0.86	0.71–1.15	0.75–0.88	0.75–1	1.07	0.8	0.75–1	
	0.86	0.87 ± 0.12	0.83 ± 0.07	0.85 ± 0.13	1.07		0.86 ± 0.11	
	1	11	3	3	1	1	7	
Distance to pharynx as % of body length	23.3–32.8	27.7–31.3	26.9–29.4	26.2–26.4	23.9–30.6	ND	ND	
	27.3 ± 4.89	29.4 ± 1.3	28.1 ± 1.79	26.3 ± 0.17	27.6 ± 2.81			
	3	10	2	2	4			
Body width: oral sucker width	0.88	0.68–1.00	0.73–0.80	0.63–0.89	0.58	0.56	0.5–0.69	
	0.88	0.84 ± 0.11	0.77 ± 0.04	0.73 ± 0.14	0.58		0.58 ± 0.06	
	1	11	3	3	1	1	7	

Measurements (μm) as range, mean ± standard deviation and number of specimens. Forebody was measured as the distance between the anterior extremity of the body and the anterior margin of the ventral sucker. Sucker width ratio is given with oral sucker as one. Distance to pharynx was measured from distal end of oral sucker to proximal end of pharynx. Body width:oral sucker width ratio is given with body as one.

Scomber australasicus are omnivores that feed primarily upon pelagic ascidians, pyrosomes and salps, and, to a lesser degree, on fish and crustaceans (Bulman *et al*., 2001). *Scomber japonicus* and *S. australasicus* also feed on the siphonophore *Chelophyes appendiculata* (Takano, 1954) and *S. scombrus* feeds on the hydromedusan *A. digitale* (Runge *et al*., 1987). Given the broad diet range (including gelatinous zooplankton) of *S. australasicus* and its congeners, it seems likely that *S. australasicus* also feeds upon *A. eurodina*. This information suggests that *A. eurodina* is a genuine intermediate host for *O.* cf. *kahawai*. The range of animals in which *Opechona* metacercariae and sexual adults have been found suggests there may be other second intermediate (and definitive) hosts in the Pacific. Gastropods from the superfamily Buccinoidea, *Costoanachis avara*, *Nassarius pygmaeus* and *Astyris lunata* are the first intermediate hosts of *O. pyriforme*, *O. bacillaris* and *O. cablei* (Stunkard, 1969; Køie, 1975; Stunkard, 1980b, respectively). Species of *Nassarius* are found along much of the Australian coastline (ABRS, 2020), so would be appropriate targets for further investigation into the life cycle of *O.* cf. *kahawai*.

Adult *O. kahawai* have been found in the guts of *Arripis* (Bray & Cribb, 2003) and *S. lalandi* (Hutson *et al*., 2007). These are large, predatory fish that may seem unlikely to prey upon gelatinous zooplankton. Thus, it seems likely that they become parasitized by feeding upon fish that eat gelatinous zooplankton so that the life cycle involves four hosts. In Australian waters, the diet of *S. lalandi* includes *Trachurus* sp. and *S. australasicus* (Ward *et al*., 2008). *Trachurus* species are definitive hosts of many *Opechona* species, including *O. bacillaris* (Kovaleva, 1963 and Nikolaeva & Kovaleva, 1966, both cited in Bray & Gibson, 1990; MacKenzie *et al*., 2004), *Opechona* species (Machida & Uchida, 1990), *O. pyriforme* (MacKenzie *et al*., 2004) and also host metacercariae of *O. bacillaris* (Gaevskaya & Kovaleva, 1982, cited in Bray & Gibson, 1990). *Trachurus* species are a main prey item of *Arripis trutta* in south-eastern Australia (Hughes *et al*., 2013). *Trachurus* species are known to associate with jellyfish (Masuda *et al*., 2008; Kondo *et al*., 2016) and, thus, seem a likely pathway for *O. kahawai* to reach *S. lalandi* and species of *Arripis*.

The definitive hosts of *Opechona* species in the north-east Atlantic are predominately fishes of the family Scombridae: *S. australasicus*, *S. japonicus*, *S. scombrus*, *Rastrelliger brachysoma* and *Rastrelliger kanagurta* (see Bray & Gibson, 1990 for references); but also include fishes from at least 13 other families (see Bray & Gibson, 1990). Interestingly, the other *Opechona* species found in Australia, *O. austrobacillaris*, is known from the predatory fish *Pomatomus saltatrix* (Bray & Cribb, 1998). Whilst gelatinous zooplankton have been found in their stomach contents, stable isotopes have shown their diet to be dominated by fish and squid (Cardona *et al*., 2012) and that their main prey are *Scomber* species. As scombrids have been categorized as a major definitive host of *Opechona* species, it is plausible that adult *O. austrobacillaris* and *O. kahawai* from *Scomber* species eaten by predatory fish have survived and thrived in the intestines of the predators *P. saltatrix* and *S. lalandi*, respectively.

Implications of findings

There have been no studies of the effects of *O.* cf. *kahawai* or *C. warehou* on their fish hosts. However, trematodes such as these, found in the intestines of their definitive hosts, are generally not considered significant pathogens (Cribb, 2005). These

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Table 4. Number of digeneans within each location of their jellyfish hosts.

		Location within jellyfish				
Digenean species	Jellyfish host species	Bell	Stomach	Oral arms	Oral pillar/disc	Tentacle
Cephalolepidapedon warehou	*Cyanea annaskala*	2		3		1
	Pseudorhiza haeckeli	43	1	72	9	
Opechona cf. *kahawai*	*Aequorea eurodina*	38				

digeneans are small in comparison to the fish studied and occurred in low intensities. Although intestinal digeneans may feed on food within the host's intestines, and not directly damage the host, they may still reduce fitness of the host fish through energy loss and increased feeding effort (Bartoli & Boudouresque, 2007). The effects of the digenean parasites may be greater upon hosts other than fish within their life cycle.

No obvious effects of the digeneans were observed on the jellyfish. This is consistent with observations by Køie (1975) on most ctenophores and hydromedusae infected by *O. bacillaris*. However, she found that very small hydromedusae *Hydractinia carnea* were unable to swim when penetrated by four cercariae. Sizes of the hydromedusae were not provided, but even large *H. carnea* medusae have a bell diameter of only 2.4 mm (Schuchert, 2008) – much smaller than the jellyfish in this study. Populations of the ctenophore *Pleurobrachia pileus* declined after heavy infection by *O. bacillaris* and didymozoid trematodes (Yip, 1981, 1984), suggesting that heavy infections can negatively affect second intermediate jellyfish hosts. The hosts most affected by *O. bacillaris* and *C. warehou* are likely to be the first intermediate hosts, which are typically castrated by digeneans (Cribb, 2005). While there are no studies on first intermediate hosts of *C. warehou*, male gastropods (*N. pygmaeus*) infected with *O. bacillaris* cercariae had a highly reduced mating organ, and infected males and females had reduced and non-functional gonads (Køie, 1975). Similarly, gastropods parasitized by lepocreadiid cercariae believed to be *Opechona* sp. were completely castrated (Averbuj & Cremonte, 2010). As prevalences of infected snails may be high (e.g. 7.4% of *N. pygmaeus* in Køie's study, up to 54.2% of *Buccinanops cochlidium* in Averbuj and Cremonte's), infections could have effects on a population level.

Conclusion

This study identified new jellyfish hosts for the lepocreadiid trematodes *C. warehou* and *O.* cf. *kahawai*. Sequences of ITS2 proved to be an effective tool in identifying digenean metacercariae from jellyfish. Results from morphology, ecological data and previous studies suggest that these jellyfish act as second intermediate hosts of these digenean species. New definitive hosts for *C. warehou* and *O.* cf. *kahawai* have been identified (*Seriolella brama* and *Scomber australasicus*, respectively) and previously reported definitive host of *C. warehou* confirmed (*Seriolella punctata*).

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Conflicts of interest. None.

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