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Description of *Tylodelphys darbyi* n. sp. (Trematoda: Diplostomidae) from the threatened Australasian crested grebe (*Podiceps cristatus australis*, Gould 1844) and linking of its life-cycle stages

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Research Paper

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Abstract

Species of the genus *Tylodelphys* (Diplostomidae) have a cosmopolitan distribution. Metacercariae of these species infect the eye, brain, pericardial sac or body cavity of fish second intermediate hosts, and the adults are found in piscivorous birds of many orders. An unnamed species of *Tylodelphys* from the eyes of bullies (*Gobiomorphus cotidianus*) was characterized molecularly and morphologically as a metacercaria in a previous study, in which it was predicted that the adult of this species would be found in the Australasian crested grebe. Two specimens of this bird became available and specimens of the unnamed *Tylodelphys* species were, indeed, found in them, confirmed by identity of genetic sequence data. Found to differ morphologically from its congeners, the new species is here described as *Tylodelphys darbyi* n. sp. Three species are closest to the new species in morphology: *Tylodelphys glossoides*, *T. immer* and *T. podicipina robrauschi*. Compared with *T. darbyi* n. sp. these three species are slightly larger and possess longer eggs. *Tylodelphys glossoides* also differs in having a wider oral sucker and *T. podicipina robrauschi* in having comma- or kidney-shaped pseudosuckers and an ovary that reaches a larger size, along with higher upper limits for body width, hind body and sucker width, holdfast and oesophagus length, and pharynx, pseudosucker and testes length and width. *Tylodelphys immer* also differs from *T. darbyi* n. sp. in having a shorter ventral sucker and the largest pseudosuckers of any *Tylodelphys* species.

Introduction

Species of the genus *Tylodelphys* Diesing, 1850 (Diplostomidae Poirier, 1886) have a cosmopolitan distribution and are found in Africa, Asia, Europe and America (see Blasco-Costa *et al.* (2017) for a comprehensive list). Metacercariae of these species infect the eye, brain, pericardial sac or body cavity of fish second intermediate hosts, and the adults are found in piscivorous birds of many orders (Blasco-Costa *et al.*, 2017).

Until recently there were only two records of species of *Tylodelphys* for Australasia: *T. podicipina* Kozicka & Niewiadomska, 1960 and *Tylodelphys* sp. (Dubois and Angel, 1972; Kennedy, 1995). The metacercaria stage of an unnamed species of *Tylodelphys* from the eyes of bullies (*Gobiomorphus cotidianus* McDowall, 1975) in the South Island of New Zealand was characterized molecularly and morphologically by us (Blasco-Costa *et al.*, 2017), in the anticipation that eventually the adult would be discovered, almost certainly in the intestine of the Australasian crested grebe (*Podiceps cristatus australis,* Gould, 1844). There were two reasons for suspecting grebes of being the trematode's final host. Firstly, Northern Hemisphere *Tylodelphys* species often use grebes as their final hosts (Dubois, 1968; Storer, 2000), and therefore it would make sense that Southern Hemisphere species do the same. Secondly, the parasite has been found only in the eyes of bullies in Central Otago lakes, and not in coastal lakes that had been sampled extensively. Therefore, the definitive host was most likely to be a fish-eating bird restricted to central lakes, ergo grebes.

The crested grebe (*P. cristatus*) has a broad distribution in Europe, Asia and Africa, but the subspecies *P. cristatus australis* is listed as Nationally Vulnerable for New Zealand. In addition, the species is considered taonga ("treasure") by Maori and is fully protected. Consequently it has proved impossible to cull these birds and they have not previously been examined for parasites. However, in January 2017 a dead individual was collected from Lake Wanaka, allowing a first investigation of the endoparasites of the New Zealand population of this bird species. As anticipated, a number of specimens resembling *Tylodelphys* species previously described were found as adults in the intestine of the grebe. Subsequently, a white-faced heron (*Egretta*

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novaehollandiae (Latham, 1790)) was also discovered dead in a nearby location and this, too, proved to be infected with specimens of *Tylodelphys* sp., and a second, infected, grebe was found a few months later.

This study aims to complete another stage of the life cycle of this diplostomid by matching it genetically with its metacercariae as previously characterized (Blasco-Costa *et al.*, 2017); to describe and name the adult on the basis of morphological features; and to distinguish it from its congeners for which the adult stage is known.

Materials and methods

Specimens

One adult male Australasian crested grebe (*Podiceps cristatus australis* Gould) was found dead at Mou Waho Island, Lake Wanaka, South Island, New Zealand (44.55°S, 169.08°E), dissected *in situ* and the viscera delivered, frozen, to the first author in January 2017. Thirty-one specimens resembling *Tylodelphys* were found in the intestine. A second grebe was found dead in Roy's Bay, Lake Wanaka (44.41°S, 169.07°E) and collected, frozen, for dissection a year later, in January 2018. The second bird contained two specimens of the worm. A white-faced heron was found freshly dead but with considerable carnivore damage at Glendhu Bay, Lake Wanaka (44.39°S, 168.59°E) in June 2018. The intestine was intact, and this contained four similar specimens. Upon dissection of the intestines, worms recovered were stored in 70% ethanol. From the first grebe, three individuals of the new species were chosen as vouchers for molecular investigation. A small piece of the anterior end of each voucher was cut off for DNA extraction. For light microscopy, specimens were stained using acetic acid iron carmine, cleared in clove oil and permanently mounted in Canada balsam. Drawings were made using an Olympus drawing tube mounted on an Olympus compound microscope. Measurements were made using the Olympus DP2-BSW application software on an Olympus BX51 compound microscope mounted with DP25 camera attachment (Olympus, Tokyo, Japan). They are presented as the range followed by the mean in parentheses, in µm unless otherwise stated. The forebody measurement was made from the anterior tip to the "raised posterior border of the more-or-less concave anterior segment" (see Dubois, 1968, p. 294). For scanning electron microscopy, the worms were washed for two hours in distilled water before being fixed overnight in 2.5% glutaraldehyde in 0.1M cacodylate buffer. They were then post-fixed in 1% osmium tetroxide for 1 h prior to being dehydrated through a gradient ethanol series, critical point dried in a CPD030 BalTec critical-point dryer (BalTec AG, Balzers, Liechtenstein) using carbon dioxide, mounted on aluminium stubs using double-sided adhesive carbon tape, and sputter coated with gold/palladium (60 : 40) to a thickness of 12 nm in an Emitech K575X Peltier-cooled high-resolution sputter coater (EM Technologies, Ashford, Kent, UK). The specimens were viewed with a JEOL 6700F field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan) at the Otago Centre for Electron Microscopy (OCEM, University of Otago, New Zealand).

Amplification and sequencing of DNA

Genomic DNA was extracted from worm tissue in 200 µl of a 5% suspension of Chelex® in deionised water and containing 0.1 mg/ml proteinase K followed by incubation at 56°C for 5 h, boiling at 90°C for 8 minutes, and centrifugation at 14,000 g for 10 minutes. Only one trematode from the first grebe (out of three specimens) amplified successfully for a partial fragment of the ITS1 gene, using primers M1780F: 5′-ACA CCG CCC GTC GCT ACT A-3′ and M5.8R: 5′-GGC TGC GCT CTT CAT CGA CA-3′ (Galaktionov *et al.*, 2012). Polymerase chain reaction (PCR) amplifications were performed in a final volume of 25 µl, comprising 5 µl of MyTaq™ Red 5× reaction buffer (Bioline), 1 µl of each primer (10 µm), 0·1 µl (1 U) of MyTaq™ Red DNA Polymerase, and 5 µl of extracted genomic DNA. Thermocycling conditions used for ITS1 amplification were as follows: denaturation (95°C for 5 minutes); 35 cycles of amplification (94°C for 50 s, 54°C for 50 s, 72°C for 1 minute 20 s); 4 minute extension at 72°C. PCR amplicons were purified prior to sequencing using exonuclease I and shrimp alkaline phosphatase enzymes (Werle *et al.*, 1994). Amplicons were cycle-sequenced from the forward strand only using PCR primer, employing BigDye® Terminator v. 3.1 Ready Reaction Cycle Sequencing Kit, alcohol-precipitated, and run on an ABI 3730XL Analyser (Applied Biosystems, Foster City, CA, USA). Specimens from the heron were degraded and did not amplify successfully.

Results

Diplostomidae Poirier, 1886; *Tylodelphys* Diesing, 1850;

Tylodelphys darbyi n. sp. (figs 1 and 2; table 1)

Synonym. *Tylodelphys* sp. (Lagrue and Poulin, 2015; Maceda-Veiga *et al.*, 2016; Stumbo and Poulin, 2016; Blasco-Costa *et al.*, 2017; Chaudhary *et al.*, 2017a).

Description (based on 16 stained and mounted gravid specimens). Body linguiform, indistinctly bipartite and tegument aspinous, 1219–1529 (1358) long. Forebody slightly spatulate, longer than hindbody, 765–1091 (950) × 357–506 (446) at level of holdfast organ. Hindbody conical, 329–495 (405) long. Oral sucker subterminal, 78–99 (91) × 82–105 (90). Ventral sucker equatorial to body length, 100–120 (110) × 82–125 (108); always larger than oral sucker (VS : OS width ratio 1 : 0.7–1.0 (1 : 0.8)). Distance between anterior border of ventral sucker and anterior extremity 440–596 (518). Pseudosuckers well developed, elongated oval, 166–209 (186) × 56–88 (73), 11–16 (14)% of body length. Holdfast organ elliptical, muscular, strongly protrusive in lateral view: 198–299 (241) × 177–271 (241), 15–20 (18)% of body length. Distance between ventral sucker and holdfast organ 18–83 (43). Prepharynx absent; pharynx elliptical, 65–94 (82) × 55–65 (58). Oesophagus short, 0–37 (22). Copulatory bursa eversible at an angle postero-ventrally, 110–231 (156) × 130–205 (172); large genital cone; genital pore subterminal. Testes tandem, extended transversally in a horseshoe shape with two ends facing ventrally; anterior testis, 80–112 (96) × 288–367 (331); posterior testis 67–98 (80) × 236–315 (287). Ovary transversely oval or reniform, median or slightly left of mid-line, pretesticular, 69–96 (80) × 85–125 (108). Vitellarium follicular, in fore- and hindbody; in forebody extend less than halfway between ventral sucker and intestinal bifurcation, follicles form six longitudinal bands in forebody and are more concentrated around holdfast organ; two bands of follicles around lip of hindbody and a few posterior to testes. Uterus containing up to 7 eggs, 72–85 (79) × 47–57 (53), 5–7% (6%) of body length.

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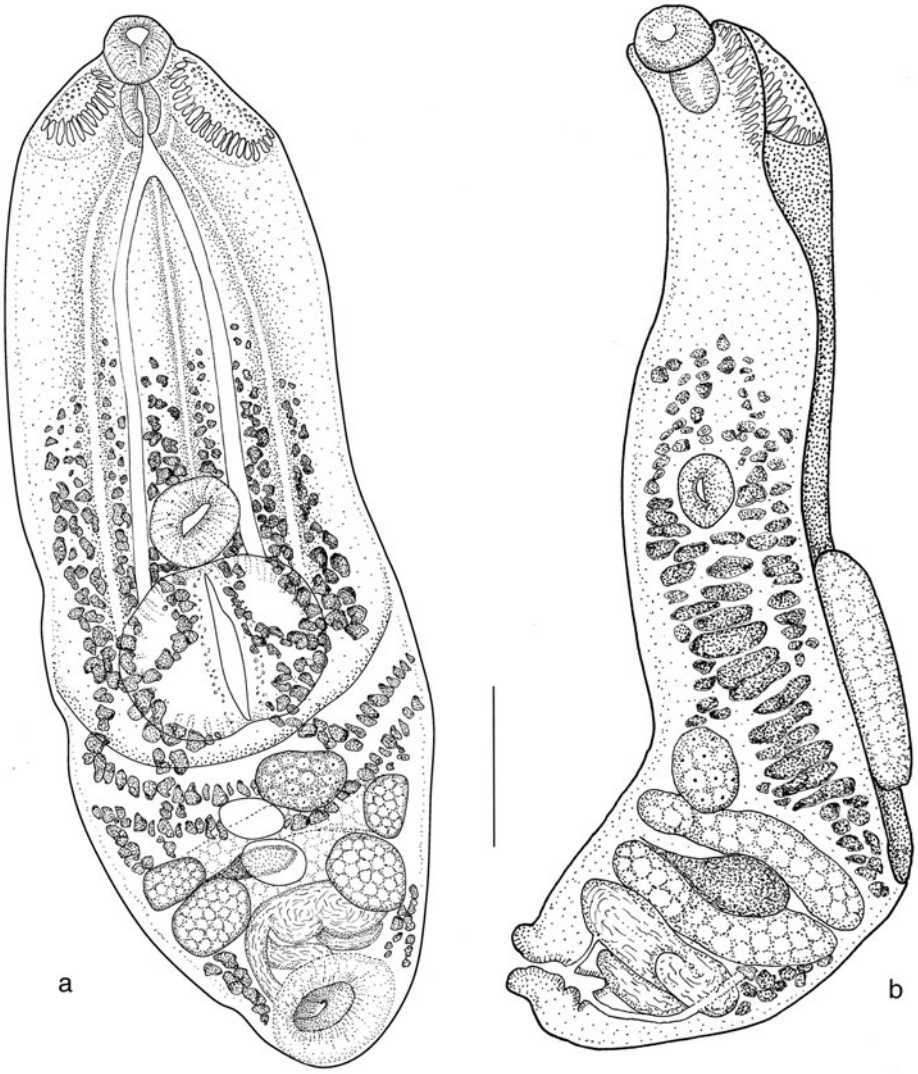

Fig. 1. (a) *Tylodelphys darbyi* n. sp. holotype (MHNG-PLAT-121211) ex *Podiceps cristatus australis* Gould, ventral view. (b) *T. darbyi* n. sp. paratype (MHNG-PLAT-121212) ex *P. cristatus australis*, lateral view. Scale bar 200 μm.

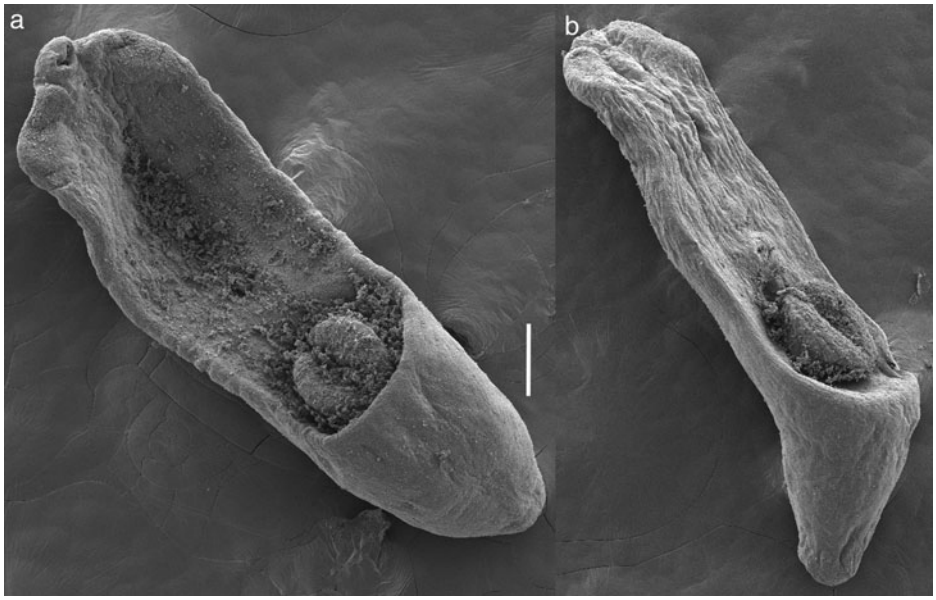

Fig. 2. Scanning electron micrographs of (a) *Tylodelphys darbyi* n. sp. paratype ex *Podiceps cristatus australis* Gould, ventral view, and (b) *T. darbyi* n. sp. paratype ex *P. cristatus australis*, oblique view. Scale bar 100 μm.

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Table 1. Measurements of *Tylodelphys darbyi* n. sp. from two grebes (n = 16) and a heron (n = 3), with comparisons to the three species that are closest morphologically: *T. immer*, *T. glossoides* and *T. podicipina robrauschi*, for which measurements are taken from Dubois (1968).

Species name	*T. darbyi* n. sp.		*T. glossoides*	*T. immer*	*T. podicipina robrauschi*
1st host	Not known	Not known	Not known	Not known	Not known
2nd host	*Gobiomorphus cotidianus*	*G. cotidianus*	Not known	Not known	*Dallia pectoralis, Cottus cognatus*
Definitive host	*Podiceps cristatus australis* (Australasian crested grebe)	*Egretta novaehollandiae* (White-faced heron)	*Gavia arctica* (Black-throated diver)	*Gavia immer* (Common loon)	*Podiceps grisegena holboellii* (Red-necked grebe)
Locality	Wanaka NZ	Wanaka NZ	Switzerland	N. America	Alaska
Body length	1219–1529 (1358)	940–1213 (1044)	1500–1800	1050–1800	850–1620
Width at tribocytic organ	357–506 (446)	–	450–480	320–580	400–800
Forebody length	765–1091 (950)	652–869 (731)	1178*	690–1140	550–990
Hindbody length	329–495 (405)	313–429 (358)	408*	330–790	260–630
Oral sucker length	78–99 (91)	73–87 (80)	90–108	72–120	70–135
oral sucker width	82–105 (90)	71–89 (82)	108–123	80–115	57–120
Pharynx length	65–94 (82)	52–71 (63)	72–90	60–89	52–122
Pharynx width	55–65 (58)	39–58 (48)	65	48–70	52–102
Ventral sucker length	100–120 (110)	89–104 (96)	100–108	70–103	80–120
Ventral sucker width	87–125 (108)	95–104 (101)	108–117	80–122	100–155
Holdfast organ length	198–299 (241)	176–267 (208)	225–245	180–315	120–320
Holdfast organ width	177–271 (241)	164	270	210–395	110–265
Oesophagus	0–37 (22)	–	30	5–52	0–50
Pseudosucker length	166–209 (186)	139–190 (158)	200–225	180–280	150–245
Anterior testis length	80–112 (96)	62–85 (76)	–	90–220	80–210
Anterior testis width	288–367 (331)	210–223 (217)	315	250–380	270–610
Posterior testis length	67–98 (80)	53–62 (56)	–	90–195	100–240
Posterior testis width	236–315 (287)	189–199 (100)	270	215–350	250–530
Ovary length	69–96 (80)	54–74 (64)	–	95–115	85–117
Ovary width	85–125 (108)	87–113 (100)	–	80–145	110–170
Egg number	1–7	1–4 (2)	6*	3–17	25
Egg length	72–85 (79)	70–81 (74)	94–108	85–104	85–98
Egg width	47–57 (53)	50–52 (51)	52–69	54–68	54–63

Egg shape (l/w)	1.5	1.5	1.7	1.6	1.6
Distance ventral sucker–anterior end	440–596 (518)	365–399 (382)	664*	519*	509*
Distance ventral sucker–holdfast organ	18–83 (43)	–	34*	10*	36*
Ratio hindbody/ forebody length	0.32–0.58 (0.43)	0.47–0.51 (0.49)	0.35*	0.45–0.76	0.41–0.66
Ratio sucker width (oral/ ventral)	0.7–1.0 (0.84)	0.68–0.89 (0.81)	1.0*	1.16*	1.03–1.54 (1.21)
Ratio body/p-sucker length	6.33–8.54 (7.32)	6.38–7.04 (6.64)	7	5.8–8.3 (6.6)	5–9 (6.7)
Ratio body/holdfast organ length	5.0–6.65 (5.69)	4.65–5.34 (5.08)	6.51*	5.54*	9.5*
Ratio forebody/holdfast organ length	3.27–4.79 (3.99)	3.33–3.70 (3.55)	4.7*	3.3*	5.1*
Ratio oral sucker/pharynx length	0.91–1.38 (1.12)	1.23–1.40 (1.31)	1.2*	1.4*	1.0*
Pharynx/oral sucker length	0.72–1.1 (0.9)	0.71–0.82 (0.76)	0.83*	0.72*	1.0*
Ratio body/egg length	15.24–18.82 (17.0)	11.60–17.08 (14.23)	16.9*	17.1*	16.0*

*Measurements and ratios are from illustrations in Dubois (1968).

Type host. Australasian crested grebe (*Podiceps cristatus australis* Gould, 1884) (Aves, Podicipediformes, Podicipedidae).

Other host. White-faced heron (*Egretta novaehollandiae* (Latham, 1790)) (Aves, Pelicaniformes, Ardeidae).

Type locality. Mou Waho Island, Lake Wanaka, New Zealand (44.55°S, 169.08°E).

Other locality. Roy's Bay and Glendhu Bay, Lake Wanaka, New Zealand (44.39°S, 168.59°E).

Site of infection. Intestine.

Intensity. 31 and 2 in two grebes, 4 in a single heron.

Type specimens. Holotype MHNG-PLAT-121211; paratypes MHNG-PLAT-121212–14 (Muséum d'histoire naturelle, Geneva), OMNZ IV-101754-7 (Otago Museum, Dunedin).

GenBank accession number. KU588152.

Etymology. The species is named for John Darby, whose tireless efforts have almost single-handedly conserved the Australasian crested grebe population in New Zealand, and who kindly provided our bird specimens.

Remarks

The ITS1 fragment amplified (550 bp) was used in a BLASTn search (http://blast.ncbi.nlm.nih.gov/) in GenBank to confirm its identity with the GenBank sequence KU588152, from *Tylodelphys* sp. metacercaria of Blasco-Costa *et al.* (2017). The adult diplostomid described above is genetically identical to *Tylodelphys* sp. of Blasco-Costa *et al.* (2017) found as a metacercaria in the eyes of bullies (*Gobiomorphus cotidianus*) from New Zealand. Amplification of the samples was not straightforward due to DNA degradation of the specimens after the birds' death. However, a 550 bp section of the ITS1 gene was sequenced, which, notwithstanding some ambiguous sites, was closest to the New Zealand *Tylodelphys* sp. sequence in a BLASTn search in GenBank. Manual base calling was possible for some of the ambiguous sections, and all readable sections were identical to the *Tylodelphys* sp. metacercaria. A phylogeny depicting its relationship to other *Tylodelphys* spp. has been presented in Blasco-Costa *et al.* (2017).

The specimens from the white-faced heron (n = 3 mounted and measured) are, on average, smaller than those from the grebe, with concomitantly smaller measurements throughout, but the metrics overlap (see table 1). In addition, the ratios of body length to various organs and that of the two suckers are very close to those found in the grebe specimens. Combined with the fact that these birds came from the same site, consume a very similar diet and that broader molecular screening of metacercariae from the lake in Blasco-Costa *et al.* (2017) has only ever found a single metacercarial type in the lake, we are led to conclude that they are the same species. In order to follow good practice in the field, if further heron specimens ever become available it will be desirable to confirm their identification using molecular data.

The studied specimens are medium sized trematodes that conform to the diagnosis of genus *Tylodelphys* by having an indistinctly bipartite body, conical hindbody shorter than forebody, well-developed pseudosuckers, non-trilobate anterior extremity, symmetrical anterior testis wider than posterior, and a copulatory bursa enclosing a small genital cone with a hermaphroditic duct opening terminally (Kozicka and Niewiadomska, 1960; Niewiadomska, 2002).

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Of the 29 nominal species of *Tylodelphys*, 21 have been described at the adult life stage (Blasco-Costa *et al.*, 2017). The remaining eight were described only as metacercariae; of these, six are considered *insertae sedis* until such time as the adults are available to describe or until molecular evidence is available for comparative purposes (see Blasco-Costa *et al.*, 2017). Two species described as metacercariae have been characterized morphologically and molecularly, and are thereby considered valid: *T. jenynsiae* (Szidat, 1969) and *T. cerebralis* (Chakrabarti, 1968) (Chakrabarti, 1968; Szidat, 1969; Locke *et al.*, 2015; Chaudhary *et al.*, 2017b). In addition to these nominal species some 13 unnamed species have been characterized morphologically (Dubois, 1978) and/or molecularly (Locke *et al.*, 2015; Chaudhary *et al.*, 2017a,b).

No obvious morphological feature allows unambiguous distinction of the new species from all other congeneric species, thus a combination of morphometric features is required to accurately distinguish the species. Compared to all other *Tylodelphys* species described as adults, *T. aegyptius* Quaggiotto & Valverde, 1992, *T. brevis* Drago & Lunaschi, 2008, *T. mashonense* (Beverley-Burton, 1963), *T. rauschi* (Singh, 1956) and *T. xenopi* (Nigrelli & Maraventano, 1944) have overall a shorter body and smaller suckers, pharynx, holdfast, ovary, and pseudosucker length than *T. darbyi*. *Tylodelphys aztecae* Garcia-Varela *et al.*, 2015 is also shorter in body length, with a smaller pharynx than *T. darbyi* but with a longer oesophagus. *Tylodelphys americana* (Dubois, 1936), *T. clavata* (von Nordmann, 1832) and *T. duboisilla* (Mehra, 1962) overlap in body size with the new species but differ in having smaller suckers and pseudosuckers. *Tylodelphys strigicola* Odening 1962 differs from *T. darbyi* in having a longer body but shorter suckers, pharynx and pseudosuckers, and wider testes. *Tylodelphys chandrapali* Jain & Gupta, 1970, *T. darteri* Mehra, 1962 and *T. elongata* (Lutz, 1928) have a much longer body and eggs than *T. darbyi*, as well as wider testes. *Tylodelphys chandrapali* and *T. darteri* also have a larger ovary than *T. darbyi*, whereas *T. darteri* and *T. elongata* have a shorter ventral sucker than the latter. The larger bodies and shorter pseudosuckers lead to considerably higher ratios of body length to pseudosucker length (7.3 on average in *T. darbyi* versus 10.0–22.3 in the other three species). *Tylodelphys adulta* Lunaschi & Drago, 2004 and *T. conifera* (Mehlis, 1846) have the same range in body length as *T. darbyi* but their ventral sucker is shorter and their eggs longer. *Tylodelphys adulta* is also characterized by a covering of fine spines and vitellaria that barely extend beyond the ventral sucker, along with the smallest eggs reported. *Tylodelphys excavata* (Rudolphi, 1803) is almost twice as long as *T. darbyi* but most of its organs are similar in size to those of *T. darbyi*, except for a shorter ventral sucker and longer oesophagus. Thus, ratios between suckers, pseudosucker length to body length and egg length to body length are much higher for *T. excavata* than for *T. darbyi*. *Tylodelphys podicipina* has a slightly longer body, wider testes and a larger ovary than *T. darbyi*, as well as a larger egg to body length ratio. *Tylodelphys excavata spinnata* (Gupta, 1962) differs from *T. darbyi* in having a globular or subglobular ovary that is situated at the very edge of the body as opposed to near the midline.

Tylodelphys glossoides (Dubois, 1928), *T. immer* Dubois, 1961 and *T. podicipina robrauschi* Dubois, 1969 most closely resemble *T. darbyi*, although these three species are slightly longer and have longer eggs. Comparative measurements are given in table 1. *Tylodelphys glossoides* also differs from *T. darbyi* in having a wider oral sucker, and from *T. podicipina robrauschi* in having comma- or kidney-shaped pseudosuckers and an ovary that reaches a much larger size, along with higher upper limits for body width at holdfast organ, hind body and sucker width, holdfast and oesophagus length, pharynx, pseudosucker and testes length and width. *Tylodelphys immer* also differs from *T. darbyi* in having a shorter ventral sucker and the largest pseudosuckers of any species. Of the three species most closely resembling *T. darbyi*, molecular sequences were available only for *T. immer*, which appeared as a sister species to *T. darbyi* in the phylogenetic reconstruction of Blasco-Costa *et al.* (2017) using the *cox* 1 marker, and as part of the same unresolved clade in which *T. darbyi* was placed in the ITS phylogenetic tree.

Apart from *Tylodelphys darbyi* n. sp. as described above, several other intestinal parasites were recovered from both the grebes and the heron. Both grebe specimens were infected with a large number of *Contracaecum* sp. and one or two capillarid nematodes, along with hundreds of individuals of *Cryptocotyle* sp., over a hundred echinostomes and a small hymenolepidid cestode. The heron harboured many hundreds of *Contracaecum* sp., a single capillarid, many tiny trematodes in poor condition, and many echinostomes. Both bird species contained thousands of encysted and newly excysted *Apatemon* sp. "jamiesoni" (Blasco-Costa *et al.*, 2016) in their stomachs. This latter strigeid trematode is more usually found in water fowl, and the fact that not a single mature individual was found in the grebes or heron suggests that these birds are unsuitable hosts for this unnamed species of *Apatemon*, which still awaits a formal description of the adult.

Discussion

DNA sequence data supported conspecificity of the metacercariae from bullies and the adult from the grebe, even if the quality of the data was not ideal. This reiterates the usefulness of molecular data to elucidate the complex life cycles of helminth parasites (Cribb *et al.*, 1998; Pina *et al.*, 2009; Locke *et al.*, 2011; Chibwana *et al.*, 2015; Selbach *et al.*, 2015). Genetic matching stands as the preferred option now that it is less expensive and easier than in previous years, more reliable than morphological matching, and quicker and simpler than experimental infections as well as avoiding ethical complications (Blasco-Costa and Poulin, 2017). Furthermore, knowledge about the larval stages of these species allows the consideration of additional morphological characteristics for discriminating species.

Kozicka and Niewiadomska's (1960) emendation of the diagnosis of *Tylodelphys* includes "oral sucker larger than ventral" (also Niewiadomska, 2002). This is not the case in all species according to the original descriptions and redescriptions in the literature. In fact there is no pattern for the ratio of oral sucker to ventral sucker either in length or in width. Further, in individual species the ratio of the width of the suckers may be > 1 and the ratio of length < 1, or vice versa. In most cases the ratio is approximately equal.

The only recorded gastropod families serving as first intermediate host for species of *Tylodelphys* are Lymnaeidae and Planorbidae. The first intermediate host of the new species remains elusive, but ongoing searches are being made for cercariae and sporocysts in lymnaeid and planorbid gastropods from appropriate bodies of water. In Lake Wanaka, the known species of these families are the endemic *Austropeplea tomentosa* (Pfeiffer, 1855), a species of *Gyraulus*, probably the native *G. corinna* (Gray, 1850) (Pullan *et al.*, 1972; Rind, 1980; Featherston and Mcdonald, 1988), and the introduced *Lymnaea stagnalis* (L., 1758) and *Radix* sp. The presence of these snails, though

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not published, is confirmed from regular surveys by colleagues working on the fauna of Lake Wanaka (N. Davis, C. Selbach, pers. comm.). Although *A. tomentosa* is a known host for several trematodes, including important schistosomes and *Fasciola hepatica* (L.), no diplostomoid furcocercaria has been reported (Featherston and Mcdonald, 1988; Davis, 1998). *Gyraulus* sp. does not appear to be infected by trematodes in this lake (Featherston and Mcdonald, 1988). This suggests that one of the introduced snail species is the first intermediate host of *T. darbyi* n. sp.

Lymnaea stagnalis was introduced to New Zealand from Great Britain in 1864 (Thomson, 1922). The species of *Tylodelphys* native to Britain is *T. clavata*, which is not closely related to *T. darbyi* n. sp. (see phylogenetic trees in fig. 3 of Blasco-Costa *et al.*, 2016). This makes it likely that, rather than arising in New Zealand by way of the introduced lymnaeaid, *Tylodelphys darbyi* n. sp. came with the grebe host from Australia. There are no named records of *Tylodelphys* from Australia, but an unnamed species was reported in the eyes of eels (*Anguilla reinhardtii*) from Awoonga Dam, Queensland, a site that harbours Australasian grebes (Gladstone Area Water Board, Native Flora and Fauna and Fishing pamphlet), although we have no data listing the snail species present.

Crested grebes arrived in New Zealand over 10,000 years ago and occasional vagrants are still recorded (Jensen and Snoyink, 2005). They are now restricted to a number of lakes in the South Island, and a survey of 2005 found by far the largest numbers in Lake Hayes, Otago, and Lake Heron, Canterbury. Not surprisingly, prevalence of *Tylodelphys darbyi* n. sp. metacercariae is nearly 100% in common bullies in Lake Hayes (Blasco-Costa *et al.*, 2016). The white-faced heron, conversely, first arrived in New Zealand in 1868 (Carroll, 1970). Unravelling the complete life cycle of *Tylodelphys darbyi* n. sp. will be necessary to get a hint of whether this parasite may be considered native to New Zealand (as native as its grebe definitive host may be) or its presence in the country is due to the fortuitous encounter of a suitable combination of native and introduced hosts.

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